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Editorial office

Acta Physiologica Scandinavica
Karolinska Institutet
S 104 01 Stockholm

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Protein Concentration in Interstitial and Lymphatic Fluids from the Subcutaneous Tissue

By

GIANFRANCO RUTTI and KARL E. ARFORS

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Abstract

RUTTI, G. and K. E. ARFORS: Protein concentration in interstitial and lymphatic fluids from the subcutaneous tissue. *Acta physiol. scand.* 1977 99 1-8

The protein content of interstitial fluid and lymph from the same tissue was determined, fluid samples of similar volumes being taken from the subcutaneous tissue of rabbits by microprocedures. In the same lymph was collected from lymphatic vessels of 30-100 μ m. An electrophoretic technique with polyacrylamide gels in glass capillaries was used for the analysis of proteins. The gels were stained and then scanned on a microscope equipped with a moving table and photomultiplier. The area under each protein band was calculated from the recorded densitograms. The method required only a few microlitres of fluid for analysis. Using solutions of known protein concentrations the relationship between the amount of protein and the area under the densitogram band was investigated. This relationship was found to be linear, thus making possible to quantify the protein content of both interstitial fluid and lymph samples. The interstitial fluid/plasma concentration ratios for albumin, transferrin, globulins and total proteins were 0.42, 0.41, 0.42 and 0.37. The albumin/globulin ratio in interstitial fluid was found to be 1.60 of the plasma value although the values obtained for lymph were smaller to those found for interstitial fluid. The similarity between the protein concentration of interstitial fluid and lymph indicated that the endothelial lining of the terminal lymphatics did not restrict the passage of macromolecules into the lymphatics.

Key words: Interstitial fluid, lymph, proteins, subcutaneous tissue, permeability

According to the classical Starling-Landis concept, capillary fluid balance is maintained by dynamic interaction between hydrostatic and osmotic forces acting across the capillary wall. These forces are considered to be of almost equal magnitude, therefore cancelling each other out. Efforts have been made during recent years to measure the numerical values of the forces, but Wiederhielm (1971) has pointed out that the literature on capillary fluid balance still contains a number of apparently conflicting results.

One of these conflicts results from the fact that while direct measurements of hydrostatic pressure (Richardson and Zweifach 1970) have been made possible by sensitive micropressure devices (Intaglietta *et al.* 1970), the bulk of our information about interstitial fluid osmotic pressure is derived from measurement of lymph protein concentration. Experimental evidence that tissue fluid and lymph are identical in their protein composition is still lacking. Moreover, in recent reports (Casley-Smith and Bolton 1973; Casley-Smith

1976) it has been suggested that as a result of lymphatic compression, lymph is ultra-filtrate through the endothelium thus increasing the protein concentration of the lymph as compared to the interstitial fluid concentration.

Direct measurements of interstitial fluid protein concentration are difficult since only minute amounts of fluid are available by sampling from connective tissue, and adequate analytical methods for protein determination in such small volumes have not been available until now. Indirect estimates of interstitial fluid protein concentration have been made by determination of the partition of protein between plasma and the tissue space (Benson *et al.* 1955 Walker *et al.* 1960 Studer and Potchen 1968). These measurements, however, yield results which are intrinsically an expression of the average body or organ protein concentration thus making comparison with lymph protein concentration difficult.

In the present investigation a technique making it possible to analyse the protein content of samples in the nanolitre range has been used to determine the protein concentration both in interstitial fluid and lymph from the same tissue. The method consists of micropuncture for the collection of lymph and tissue fluid and an electrophoretic technique for the quantitative analysis of proteins in nanolitre samples of fluid.

Materials and methods

Protein determination

The protein content of interstitial fluid, lymph and plasma was determined by the disc-electrophoresis method of Ornstein (1964) and Davis (1964). A micro-scale modification, and the practical details, have been extensively described by Smeds (1969). Certain further modifications were, however, made before this study. In brief, the electrophoresis was run in small glass capillaries of 3 μ l. vol. (Drummond Sci. Co. Broomall, Penn. U.S.A.) with an id. 200 μ m containing a spacer and a running gel of polyacrylamide. The concentration used was 5% in the spacer and 20% in the running gel. These concentrations were double the ones used by Smeds (1969) and resulted in increased resolution of the different proteins.

Ammoniumperoxysulfate was used instead of riboflavin because it leads to more uniform cross-linking in the polyacrylamide. The pulsed voltage over the gel was kept at 175 V with a pulse rate of 500 Hz and a duty-cycle of 25%. After the run the gel was pushed out of the capillary and placed in a solution of 20% sulfosalicylic acid for 15 min. Treatment of the gel with sulfosalicylic acid was performed to make the binding of Coomassie Blue to the proteins more stable. After staining for 15 min a 0.2% solution of Coomassie Blue the gel was washed several times in a solution of acetic acid, methanol and distilled water (1:5:10) and stored for a further 24 h in the same solution.

A photomicrograph of the gel is shown in Fig. 1. A total of 15 protein bands were stained but only albumin, transferrin and α_2 macroglobulin identified. The optical density of the stained protein bands was measured with a LEITZ ORTHOLUX microscope, fitted with a photomultiplier (EMI KNOTT Type 6094 A, Knott Elektronik, Munich, West Germany). A logarithmic integrator Recorder (Viatron N.V. Dieren, Holland) gave recordings of protein extinction. For measurement the gel was placed in a slit (5.0 \times 0.4 mm) made in a brass plate fixed to a plexiglass plate. A cover glass was placed over the slit to keep the gel wet during measurement. The gel was scanned at 6 mm/min using a powered microscope table. An adjustable slit in the light path of the microscope permitted measurement of specified widths of the gel. A yellow filter (LEITZ 510) was also placed in the light beam. A densitogram is shown in Fig. 2.

To make quantitative measurements of the different proteins in the gel, the relationship between their areas under the curve and the amount of protein was investigated with solutions of standard human serum proteins (Boehringerwerk AG West Germany) of known concentrations. The volumes of standard solution used varied between 1 μ l and 15.0 μ l.

Sampling of interstitial fluid and lymph

The experiments were performed on 12 rabbits. The details of the micropuncture technique used for collection of interstitial fluid have been described previously (Rutli and Arfors 1976).



Fig 1 Photomicrograph of polyacrylamide gel electrophoresis of an interstitial fluid sample after staining with Coomassie Blue. The volume of the sample was 8.4 μ l.

In summary after careful dissection of the epidermal layer of the rabbit's hindleg, the subcutaneous tissue was exposed. In the dissected area the macrocirculation was readily visible and lymphatics of 30 to 100 μ m diameter could occasionally be observed. Lymphatic vessels smaller than 30 μ m and terminal sacs could not be identified. Using a microaspirator, small oil-filled glass pipette with tip diameter of 8-10 μ m was inserted into the tissue and fluid collected by slight aspiration produced by withdrawing 4-5 ml of air with syringe connected to the pipette holder. The sample was placed in a teflon cup filled with paraffin oil so that the droplet assumed a spherical shape and its volume could be calculated by measurements of its diameter. The whole sample was then removed and placed onto the polyacrylamide gel. After electrophoresis the gel was analysed for its protein content. Hence, a total of 20 samples could be run in parallel; the analysis was carried out simultaneously with 4 lymph samples, 4 interstitial samples, 4 plasma and 8 standards for each rabbit. The volume of the standard protein solutions was determined in the same way as for the interstitial fluid samples.

Results

The relationships between the amounts of albumin, transferrin, globulins and total proteins, and the areas under absorption curves obtained from standard solutions were investigated.

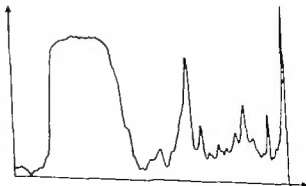


Fig 2 Densitometric recording of the separation pattern of an interstitial fluid sample. The gel was scanned on a microscope equipped with a slowly moving table and photomultiplier for measurement of the optical density.

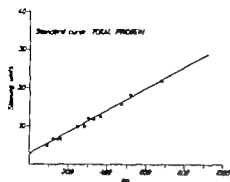


Fig. 3

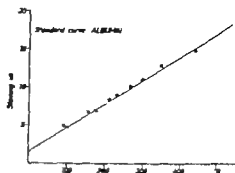


Fig. 4

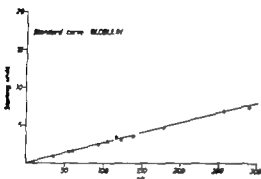


Fig. 5

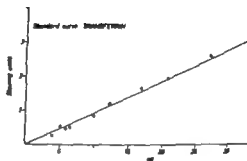


Fig. 6

Fig. 3 4 5 6. Relationship between the amount of Total Proteins, Albumin, Total Globulins, Transferrin and the amount of Coomassie Blue stain as measured from the optical density of the stained protein bands. The amount of stain is expressed as Staining Units. (For more details see text)

The results are shown in Fig. 3 4 5 and 6. The area under each curve is expressed in staining units, which are calculated for each protein fraction using the formula $SU = I/E$. I is the number of integration impulses, s is the reading given by a standard reference signal for the chart recorder which varies with the sensitivity adjustment of the recorder and E the fixed value of the standard signal (0.2).

An almost linear relation for the different proteins was found within the range used in this investigation and the coefficient of variation of the analytical procedure was calculated and found to be less than 12%. The protein concentrations of lymph (L) and interstitial fluid (IF) measured in the subcutaneous tissues of rabbits are given in Table I.

TABLE I Plasma, lymph and interstitial fluid protein concentration (g/100 ml) of the subcutaneous tissue of rabbits. The values are mean \pm S.D. of 10 animals.

	Tot. protein	Albumin	Globulins	Transferrin
Plasma	6.63	3.62	2.68	0.27
S.D.	0.58	0.56	0.40	0.03
Lymph	2.66	1.81	0.82	0.11
S.D.	0.40	0.37	0.20	0.02
Interstitial fluid	2.45	1.60	0.86	0.11
S.D.	0.41	0.30	0.19	0.02

TABLE II. Interstitial fluid and lymph/plasma proteins. Concentration ratio of rabbit subcutaneous tissue.

	Tot. protein	Albumin	Globulins	Transferrin	A/G
int. fluid	0.37	0.42	0.32	0.42	1.60
I.D.	0.06	0.08	0.07	0.09	0.22
lymph	0.34	0.46	0.30	0.41	1.70
I.D.	0.06	0.08	0.09	0.08	0.90

No significant difference between lymph and interstitial fluids was observed within the standard error of the method.

In Table II the protein concentrations of LF and lymph are compared with the respective plasma values.

The ratios for the albumin/globulin (A/G) of Table II are also expressed as ratios of the plasma values. No difference was found between LF and lymph.

The similarity between interstitial fluid and lymph is shown more directly in Table III. The values for albumin, globulins and transferrin determinations of the single experiment are given as a mean ratio between interstitial fluid and lymph concentration.

To check the reliability of the micro-electrophoretic method for quantitative determination of proteins, plasma samples from 6 rabbits were analysed for their protein content by the micro-zone electrophoretic standard method. The albumin/globulin ratios (A/G) obtained with the micro-zone electrophoretic method (I) were 1.40, 1.44, 1.25, 1.56, 0.81, 0.98 and those obtained with the micro-disk electrophoretic method (II) were 1.41, 1.50, 1.15, 1.52, 0.92, 1.00 respectively. The ratio I/II was 0.99 ± 0.06 .

Discussion

The electrophoretic method used in this investigation for both qualitative and quantitative analysis has provided a means of measuring the protein concentrations of body fluid samples of nanolitre volumes. The relatively high coefficient of variation of the method (12%) should be considered in relation to the small volumes of the samples used for protein analysis (1-15 nl). Comparative analysis of the same samples by an independent method showed no significant difference between the methods and confirmed the validity of the micro-disk electrophoretic technique. As the relationship between staining units and protein concentration was linear quantitative determinations of protein concentrations in interstitial fluid, lymph and plasma were possible. The validity of the micropuncture sampling technique for the collection of tissue fluid has been discussed elsewhere (Rutill and Ar

TABLE III. Albumin, globulins and transferrin interstitial fluid to lymph concentration ratio. The values are mean \pm S.D. of twelve rabbits.

Albumin	Globulins	Transferrin
0.91 \pm 0.12	1.09 \pm 0.21	1.01 \pm 0.17

* The values of each single rabbit are obtained from 2 to 4 interstitial and lymph fluid samples.

fors, 1976) The overall findings of the study are consistent with the view that the collected are of interstitial fluid The time required for dissection of the epidermal layer, collection of fluid samples (≈ 5 min) is relatively short, and changes due to trauma and inflammation are minimal

The concentration of total protein in the interstitial fluid and lymph from the rabbit subcutaneous tissue was found to be lower than the concentration in plasma (1.3). The values found are in agreement with those previously reported for the same tissue and species Haljam  and Freden (1970) found an interstitial fluid to plasma protein of 0.32 and Cowtice (1960) a lymph to plasma protein ratio of 0.41

Using nylon wicks to collect interstitial fluid, Aukland and Fadnes (1973) found an interstitial fluid to plasma protein ratio of 0.56 in rats which is somewhat higher than the value found in this investigation Our lower values may be due to a species difference. In studies where interstitial fluid collected using wicks were directly compared with interstitial fluid collected by micropuncture (Rutili and Arfors, to be published) no significant difference was found between the two methods.

Of the 15-16 different proteins stained, only the albumin, transferrin and total globulin concentrations were determined separately α_2 -Macroglobulin could not be quantified accurately because of its low concentration. The concentrations of these protein fractions in the interstitial fluid and in the lymph were identical (Table I) The similarity in composition between the two fluids suggests that the passage of macromolecules from the tissues to the lymphatics is not restricted by the endothelial lining of the terminal lymphatics. The morphological explanation of this no-restriction concept was given by Lenk and Burke (1968) and by Cliff and Nicoll (1970). In electron microscopic studies these workers have found "flaps and gaps" to be present in the endothelial layer of the terminal lymphatics permitting the passage of quite large molecules. Moreover they found anchoring filaments connecting the small lymphatics to the surrounding tissue, representing the mechanism responsible for opening the lymphatic capillaries in conditions of increased tissue pressure. The lack of a concentration gradient between the tissue fluid and the lymph fluid also indicates that the net transport of macromolecules between the two compartments is, at steady-state, a convective rather than a diffusive process. If this is a valid argument it will follow that the concentration of proteins in the tissue is, within their volume of distribution, evenly distributed. The capillary wall will thus represent the site of selectivity to molecular transport and the steady-state lymph/plasma ratio a measure of its permeability. Garlick and Renkin (1970) arrived at a similar conclusion as a result of kinetic studies of the passage of different macromolecules from blood to lymph. The hypothesis that lymph protein concentration is higher than interstitial fluid concentration (Casley-Smith and Bolton 1973; Casley-Smith 1976) providing the driving force for osmotic flow from interstitial fluid into lymphatic capillaries, is not supported by the present results. The idea that net transport of proteins from interstitial space into lymph by an osmotic flow occurring as a result of a concentration gradient of the same proteins, has already been shown to be incompatible with the physical laws of osmotic flow (Michel 1974)

The albumin/globulin (A/G) ratio in tissue fluid and lymph was found to be higher than the A/G ratio of plasma by a factor of 1.6-1.7. The absolute values of the A/G ratio

in interstitial fluid and lymph are not given in the results because of the large variation found in the A/G ratio of plasma between different rabbits (Table III). The mean A/G ratio of plasma was, however, 1.35 compared to a mean A/G ratio in tissue fluid of 2.20. A similar tissue fluid value (2.67) was found in the same tissue by Haljamae and Freden (1970). The higher A/G ratio of interstitial fluid relative to plasma indicates a higher permeability of vessel walls to albumin than to globulins. The transport of proteins across the microcirculation is, according to Lamm *et al* (1974) a unidirectional process. The functional morphological structure in the capillary walls through which this process takes place has been described by Grotte (1956). From studies of the transcapillary passage of dextran of increasing molecular size, he suggested the presence in the capillary wall of pores of 35–45 Å radius, excluding molecules of the size of albumin, and fewer large pores of 120–350 Å of radius through which the filtration of plasma could occur.

The effective filtration area of porous membrane available for a molecule is a function of the effective molecular radius (R_{20}) and of the pore radius. Equations for the calculation of the effective filtration area have been formulated by Renkin (1954). Using these equations and assuming a R_{20} for albumin of 35 Å and a R_{20} for globulins of 50 Å, the A/G ratio found in these experiments may be explained in terms of filtration through pores of 250–300 Å diameter in the capillary wall.

An alternative to the large pore hypothesis is vesicular transport. Endothelial vesicles were firstly described by Palade (1953) after electron microscopic observation of the fine structure of the capillary wall. Electron dense tracers such as Ferritin (R_{20} 110 Å) or Gold (R_{20} 100 Å) injected into the circulation were found to be localised within the vesicles (Karnowsky 1968) suggesting their participation in transporting material across the capillary wall. Vesicular transport is subject to steric restriction (Garlick and Renkin 1970). Assuming a vesicle radius of 250 Å (Brums and Palade 1968, Casey Smith 1968) globulins will be restricted by a factor 1–1.3 relative to albumin. This explains only in part the measured A/G ratio of 1.6–1.7 and the difference can only be explained by assuming an additional transport of albumin through the small pore system.

The identical C_1/C_2 ratio of albumin and transferrin (molecular weight 70 000 and 88 000 respectively) is of interest considering the difference in the electrical charge of the two molecules (2.1 at pH 8.6, isoelectric points 4.9 and 5.9 respectively) and suggests that the permeability of vessels to proteins is a function of molecular size.

We are grateful to M. Ove Forsberg for the skilful technical assistance in the electrophoretic analysis of this study.

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fors, 1976) The overall findings of the study are consistent with the view that the samples collected are of interstitial fluid. The time required for dissection of the epidermal layer and collection of fluid samples (≈ 5 min) is relatively short, and changes due to trauma and inflammation are minimal

The concentration of total protein in the interstitial fluid and lymph from the rat subcutaneous tissue was found to be lower than the concentration in plasma (1.3). The values found are in agreement with those previously reported for the same tissue and species. Haljam  and Freden (1970) found an interstitial fluid to plasma protein of 0.32 and Cortice (1960) a lymph to plasma protein ratio of 0.41

Using nylon wicks to collect interstitial fluid, Aukland and Fadnes (1973) found an interstitial fluid to plasma protein ratio of 0.56 in rats which is somewhat higher than the value found in this investigation. Our lower values may be due to a species difference. In studies where interstitial fluid collected using wicks were directly compared with interstitial fluid collected by micropuncture (Rutili and Arfors, to be published), no significant difference was found between the two methods.

Of the 15-16 different proteins stained, only the albumin, transferrin and total globulin concentrations were determined separately. α_2 -Macroglobulin could not be quantified accurately because of its low concentration. The concentrations of these protein fractions in the interstitial fluid and in the lymph were identical (Table I). The similarity in composition between the two fluids suggests that the passage of macromolecules from the tissues to the lymphatics is not restricted by the endothelial lining of the terminal lymphatics. The morphological explanation of this "no-restriction" concept was given by Leak and Burke (1968) and by Cliff and Nicoll (1970). In electron microscopic studies these workers have found "flaps and gaps" to be present in the endothelial layer of the terminal lymphatics permitting the passage of quite large molecules. Moreover they found anchoring filaments connecting the small lymphatics to the surrounding tissue, representing the mechanism responsible for opening the lymphatic capillaries in conditions of increased tissue pressure. The lack of a concentration gradient between the tissue fluid and the lymph fluid also indicates that the net transport of macromolecules between the two compartments is, at steady state, a convective rather than a diffusive process. If this is a valid argument it will follow that the concentration of proteins in the tissue is, within their volume of distribution, evenly distributed. The capillary wall will thus represent the site of selectivity to molecular transport and the steady-state lymph/plasma ratio a measure of its permeability. Garlick and Renkin (1970) arrived at a similar conclusion as a result of kinetic studies of the passage of different macromolecules from blood to lymph. The hypothesis that lymph protein concentration is higher than interstitial fluid concentration (Casley-Smith and Bolton 1973; Casley-Smith 1976) providing the driving force for osmotic flow from interstitial fluid into lymphatic capillaries, is not supported by the present results. The idea that net transport of proteins from interstitial space into lymph by an osmotic flow occurring as a result of a concentration gradient of the same proteins, has already been shown to be incompatible with the physical laws of osmotic flow (Michel 1974).

The albumin/globulin (A/G) ratio in tissue fluid and lymph was found to be higher than the A/G ratio of plasma by a factor of 1.6-1.7. The absolute values of the A/G ratio

Pineal Serotonin Metabolism in Non-Innervated Perinatal Glands before and after Intraocular Maturation Supersensitivity of Adrenoceptors that have never been Innervated

By

MARIA BJÖCKSTRÖM, LARS OLSON and ÅKE SENER

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Abstract

BJÖCKSTRÖM, M., L. OLSON and Å. SENER. Pineal serotonin metabolism in non-innervated perinatal glands before and after intraocular maturation: Supersensitivity of adrenoceptors that have never been innervated. *Acta physiol. scand.* 1977 99 9-18.

Transplants were made of fetal pineal glands (crown rump length, CRL, 19-30 mm) or pineal glands from adult male rats in the anterior chamber of the eye of the rat. Studies were performed with regard to the importance of the age of the donor animal (and thereby the degree of maturation and innervation of the gland to be transplanted) for the possible development of denervation supersensitivity. The transplants were cultured in medium containing ^{14}C -serotonin. Increased production of ^{14}C -N-acetylserotonin (NAcS) was used as the main criterion for β -adrenergic stimulation. 4 experimental groups are obtained by transplanting fetal or adult pineals to intact or sympathetically denervated eyes. In all 4 groups β_1 stimulation (KWD 2033 10^{-6} M) increased ^{14}C -NAcS formation. The response to β -stimulation was significantly higher in denervated fetal pineal transplants than in innervated fetal transplants and thus demonstrating β -receptor supersensitivity. It was concluded that: a) the ability to respond to β -adrenoceptor stimulation, b) increased ^{14}C -NAcS formation develops between the 18th and 20th day of gestation, c) this sensitivity also develops *in utero* as transplants that at the time of transplantation lacked the capacity to increase their ^{14}C -NAcS formation in response to treatment with β -agonist, d) denervation supersensitivity occurs in fetal transplants that become mature in sympathetically denervated eyes.

Key words: Pineal, transplants, serotonin, β -adrenergic, denervation supersensitivity, reinnervation.

The rat pineal gland metabolizes serotonin to N-acetylserotonin (NAcS) and melatonin (MEL) or 5-hydroxyindoleacetic acid (5-HIAA) (Wurtman *et al.* 1968). The activity of N-acetyltransferase (NAT), N-acetylating serotonin to NAcS (Weissbach *et al.* 1960), is controlled by β -adrenergic stimulation of the pineal nonadrenoceptor mediated through increased intracellular production of cyclic adenosine 3',5'-monophosphate (cAMP) (Klein, Berg and Weller 1970). In our previous study homologous intraocular transplants from newborn rats were analyzed 1-5-30 months following transplantation and were found to have

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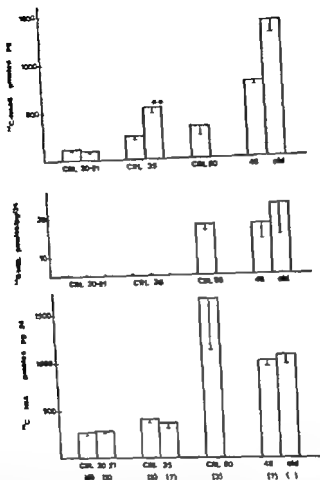


Fig 1 Production of ^{14}C -serotonin derivatives by cultured immature rat pineal glands. ^{14}C -NAC5 = ^{14}C -N-Acetylserotonin. ^{14}C -MEL = ^{14}C -Melatonin. ^{14}C -5-HIAA = ^{14}C -5-Hydroxytryptolactic acid. CRL values are crown-rump length in mm of the animals used. Empty and shaded bars indicate mean values of ^{14}C -labeled metabolite formation in unstimulated controls of KWD 2033 (10^{-6} M) stimulated glands respectively measured as picomoles of metabolite formed per pineal gland per 24 h culture period. Standard error of the mean is indicated by vertical line in bar. The number of pineal glands cultured per group presented above brackets below bars displaying ^{14}C -5-HIAA formation. ** designate groups that differ significantly ($p < 0.01$) from their respective control groups.

b. Following the preincubation period KWD 2033 (10^{-6} M) was added to some of the vessels as stated in Table 1 and Fig 2. All pineals were cultured according to the method developed by Trowell (1959), modified by Raus (1965) and first elaborated for pineal organ culture by Klein and Weller (1970). The glands were each cultured in 0.6 ml of BGJ_1 -medium, Friesen-Jackson modification (Grand Island Biological Company). Bovine serum albumin (1 mg/ml), ascorbic acid (0.1 mg/ml) and β -glutamine (0.3 mg/ml) are added on the day of the exp. The medium contained 0.25 mM ^{14}C -5-hydroxytryptamine (serotonin) creatinine sulphate (Radiochemical Center, Amersham), specific activity 34 mCi/mmol, diluted with unlabeled serotonin (Fluka AG) to 4 mCi/mmol. No correction was feasible for the decrease in ^{14}C -serotonin specific activity due to endogenous production of serotonin from L-tryptophan contained in the culture medium. The culture chamber was continuously exposed to 95% O_2 and 5% CO_2 at 37°C. All tissues were

matured and to have become innervated by sympathetic nerves. They were responsive to external lighting conditions with pronounced increases of NAT (20-1) and hydroxyindole-0-methyl transferase (HIOMT) activities during dark hours as compared to light activities (Bäckström, Olson and Selger 1976). Similar findings have recently been reported by Moore (1975). Sympathetic denervation of the host eye eliminated the dark induced rise in HIOMT activity and greatly attenuated NAT enzyme variation (2-1). Cholinergic iris nerves have been shown to readily reinnervate eye chamber transplants that receive cholinergic fibers in their normal locus (Tsukahara, Jacobowitz and Laties 1972). The constant presence of cholinergic nerves in all host irides did not modify the dependence on an intact adrenergic innervation.

The persistent low amplitude NAT variation in denervated eye chamber pineal transplants was statistically significant. One explanation for this variation could be the development of supersensitivity to circulating catecholamines in the transplants subsequent to denervation (Cannon and Rosenbluth 1949 Trendelenburg 1966). Development of denervation supersensitivity to β -adrenoceptor stimulation in rat pineal glands has been described (Deguchi and Axelrod, 1972, 1973 Bäckström and Wetterberg 1973 Strada and Weiss 1974).

It was desired to investigate whether the degree of maturation and sympathetic innervation of the grafted pineals could influence the eventual status of indole metabolism. Would receptor supersensitivity in response to β -adrenoceptor stimulation develop in a pineal gland transplanted into a sympathetically denervated eye, at a stage prior to the sympathetic fiber development in the gland? Thus, would the receptor become supersensitive without ever having been contacted by its appropriate nerve supply?

Materials and methods

Animals. Albino rats (Sprague-Dawley) were fed food pellets and water *ad libitum* and kept one (pregnant rats) or five (cage) rats. Lights were between 06 and 18 h. Pregnant rats, used for direct organ culture of fetal pineals, were decapitated between 10 and 16 h. Uteri were quickly dissected out and kept on ice while preparations of tissues were performed. Pineal glands from fetal rats with CRL 20-21 mm, 50 mm and from 48 h old rats were each taken from one litter. Pineal gland from fetal rats with CRL 35 mm were taken from two litters. The fetal or newly born rats were decapitated and using a dissection microscope the pineal glands were freed from adherent tissue. Pineal glands were stored in ice cold medium for 1-2 min before being introduced into the culture chamber. Normal control rats were 300-350 g males, housed for 14 days on the same light-dark cycle. Host animals were sacrificed by decapitation between 9.30 and 12.30 h. The eyes were immediately removed and the transplants kept on ice during the dissection. Simultaneously processed *in situ* pineal glands were dissected out and stored in ice-cold incubation medium. All tissues were placed in the incubation chamber within approximately 3 min after decapitation.

Transplantations. Pineal glands from fetal rats with CRL 19-30 mm (18-20 days of gestation) and from adult male rats (150-200 g) were bilaterally transplanted into the anterior chamber of the eye, adult recipient male rats. Pineals were rapidly dissected out from the donors, decapsulated and introduced into the anterior eye chamber through a slit in the cornea with a modified Pasteur pipette according to Olson and Mahnfors (1970). All recipient animals (totally 53 rats) underwent unilateral sympathetic denervation by extirpation of either the left or the right superior cervical ganglion a few days before transplantation. The cholinergic innervation of the recipient iris was in all cases left intact. The taking and development of the transplants were followed *in vivo* by repeated inspections through the cornea using a stereoscopic microscope under light ether anesthesia. The postoperative time ranged from 3 to 4 months.

Organ culture. Pineal glands from fetal rats and newly born rats were preincubated for 1-2 h before the β -adrenoceptor agonist KWD 2033 (10^{-4} M) (Carlsson *et al.* 1975) was added as indicated in Fig. 1. Pineal transplants and *in situ* pineal glands from experimental and normal rats were preincubated for 4

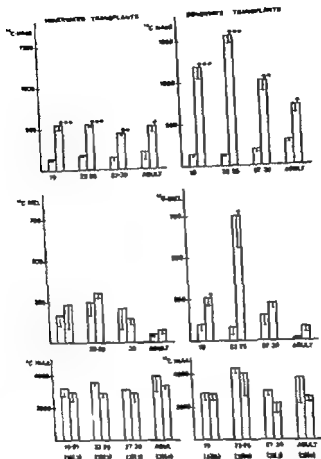


Fig. 2 Production of ^{14}C -serotonergic derivatives by cultured innervated and denervated rat pineal transplants derived from donor rats of various ages. The left part of the figure shows serotonergic formation in innervated transplants, the right shows denervated transplants. ^{14}C -NAC5, ^{14}C -N-Acetylserotonin; ^{14}C -MEL, ^{14}C -Melatonin; ^{14}C -5-HIAA, ^{14}C -5-Hydroxyindoleacetic acid. Numbers below bars give cross-hatching length (CRL) in mm of transplant donor animals. Empty and shaded bars illustrate mean values of ^{14}C -labelled metabolite formation in untransplanted controls or KWD 2033 (10^{-8} M) stimulated transplants respectively measured as picomoles of metabolites formed per transplant per 24 h culture period. Standard error of the mean is indicated by vertical line in bar. The number of pineal transplants cultured per group presented above brackets below the bars, each display ^{14}C -5-HIAA formation. * and ** designate groups that differ significantly from their respective control groups ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively).

The production of ^{14}C -5-HIAA also rose with age, reaching 25–30% of adult levels in 48 h old pineals (Fig. 1). KWD 2033 (10^{-8} M) did not affect the formation of ^{14}C -5-HIAA.

Normal mature and transplanted pineals. 1 strain pineal glands from normal rats and experimental rats (carrying bilateral eye chamber transplants) produced amounts of ^{14}C -NAC5, ^{14}C -MEL and ^{14}C -5-HIAA that did not differ statistically from each other when controls or KWD 2033 (10^{-8} M) treated groups were compared. In both types of glands KWD 2033 increased the production of ^{14}C -NAC5 (5.4- and 4.0-fold respectively) and ^{14}C -

TABLE I Production of ^3C -serotonin derivatives by cultured pineal glands and transplants.

Pineal types	Treatment		Metabolites in medium		
			III Acetyl 5-HT	Melatonin	5 HIAA
In situ normal	Control	(4)	15 \pm 32	338 \pm 67	3 930 \pm 155
	KWD 2033	(7)	828 \pm 146	656 \pm 61	2 556 \pm 473
In situ experimental	Control	(6)	167 \pm 30	294 \pm 45	3 120 \pm 168
	KWD 2033	(4)	664 \pm 215	688 \pm 138	2 904 \pm 528
All innervated transplants	Control	(16)	159 \pm 21	170 \pm 41	3 430 \pm 344
	KWD 2033	(13)	512 \pm 26	182 \pm 41	3 078 \pm 189
All denervated transplants	Control	(14)	186 \pm 27	87 \pm 22	3 406 \pm 409
	KWD 2033	(16)	1 128 \pm 96	332 \pm 79	2 943 \pm 377

Data are expressed as mean \pm standard error of the mean of picomoles ^3C -labelled metabolite formed per pineal per 24 h culture period, preceded by 1 h preincubation. N-Acetyl-5-HT = N-Acetylserotonin. 5 HIAA = 5-Hydroxyindoleacetic acid. Numbers in brackets designate the number of pineal glands or transplants cultured per group. KWD 2033 concentration was in all cases 10^{-6} M and indicate groups that differ significantly from controls of ^3C -metabolites formed as compared to their respective control groups: $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

cultured for 24 h following addition of β -adrenoceptor agonist (Bäckström and Wetterberg 1973). Media were collected and frozen at -20°C . The ^3C -labelled serotonin metabolites were separated by thin-layer chromatography as described by Klein and Nickles (1969). Radioactivity was eluted in 95% ethanol or 0.1 M HCl Instagel[®] (Packard Instrument International S.A.) or Aqueosol (New England Nuclear) were added and radioactivity determined by liquid scintillation (Packard Tri-Carb 3380, supplied with an Absolute Activity Analyzer 344).

Doses of KWD 2033 were given as the final concentration of the base in culture medium.

Statistical analyses were performed by application of Student's two-tailed *t* test for comparison of means.

Results

In vivo observations. All pineals survived transplantation into the anterior chamber of the eye regardless of the stage of development at operation. They became rapidly vascularized from the iris of the host eye. The most immature pineals (CRL 19–21 mm) were approximately 1–1.1 mm at operation and grew *in oculo* to a final size of approximately 2–1.5 mm. Pineals from CRL stages 22–25 mm and 27–30 mm were initially somewhat larger and reached the same final size as the most immature pineals. Adult pineals were approximately 2–1.5 mm already at transplantation, and did not change size *in oculo*. Denervated transplants were visibly smaller than innervated transplants regardless of donor age.

Immature pineals. Pineal glands from the most immature fetal rats (CRL 20–21 mm) formed ^3C NAcs. The production increased with maturity (Fig. 1). After addition of KWD 2033 (10^{-6} M) the pineal glands from fetal rats with CRL 35 mm and 2 day old rats significantly increased their production of ^3C NAcs (2 and 1.8 times respectively) as compared to controls ($p < 0.01$ and $p < 0.001$ respectively).

There was no production of ^3C MEL in pineal glands from fetal rats with CRL 20–21 mm or CRL 35 mm either untreated or following stimulation with KWD 2033 (10^{-6} M) (Fig. 1). Pineal glands from fetal rats with CRL 50 mm and from 2 day old rats produced minimal amounts of ^3C MEL and this production was not altered by addition of drug.

Ontogenetic development of serotonin metabolism. In earlier works pineals from fetal rats have been reported to lack responsive adenylyl cyclase which constitutes part of the proposed pineal postjunctional adrenergic receptor (Weiss 1971). However pineal glands from fetal rats with CRL 35 mm (about 20-21st day of gestation) are found to double their ^3H NACs formation in response to addition of the directly acting β -adrenoceptor agonist KWD 2033. This response was not observed in pineal glands from the most immature fetal rats with CRL 20-21 mm (about 17-18th day of gestation). NAT enzyme activity has been detected as early as 4 days before parturition. The initially high values of NAT decreased markedly around 7 days of age (Ellison, Weller and Klein 1972). Similarly in comparison with mature pineal glands a higher unstimulated level of ^3H NACs was formed around and 2 days subsequent to birth as seen in our present cultures. Our findings are compatible with the report of Weiss and Strada (1972) revealing a large increase of rat pineal phosphodiesterase activity between day 8 and 16 post partum. An increased breakdown of cAMP would lower the stimulation of postjunctional biochemical events required for maintaining NAT activity. HIOMT activity is hardly detectable during the first 10-12 days post partum in the rat pineal but then rises sharply to reach adult values at 34-50 days of age (Zweig and Snyder 1968, Snyder 1968, Klein and Lines 1969). A diurnal variation of HIOMT activity has first been detected in 39 day old rats (Klein and Lines 1969). Thus, the present absence or extremely low levels of MEL production in very young rats were in good agreement with earlier reports. Low levels of MAO enzyme activity which is responsible for the decarboxylation of serotonin to 5-HIAA, have been observed in pineals of newborn rats (Zweig and Snyder 1968, Snyder 1968). In the present organ cultures ^3H 5-HIAA formation was noted when pineals were explanted 4 days before birth, at a stage when pineals are not yet sympathetically innervated (Machado 1971). The ^3H 5-HIAA was thus formed extraneuronally probably within pinealocytes.

Transplants. Normal adult *in situ* pineal glands were cultured in the same experiment as pineal transplants and *in situ* pineal glands from experimental animals (carrying bilateral ocular transplants and submitted to unilateral superior cervical gangliectomy). Pineal glands from normal adult rats did not differ in their production of ^3H -labelled NACs, MEL or 5-HIAA as compared to the *in situ* pineal glands from experimental rats either unstimulated or β -stimulated with KWD 2033. This shows that the one remaining superior cervical ganglion supplied the *in situ* pineal glands with sufficient sympathetic nerves to ensure a normal adrenergic response. It also follows that the eye chamber transplants did not influence the activity of the *in situ* pineal gland to any significant extent.

Denervated transplants from fetal donors gave a higher increase of ^3H NACs production following β -stimulation with KWD 2033 than did innervated β -stimulated transplants taken from rats of corresponding ages indicating the development of supersensitivity to aminergic stimulation. This phenomenon was less pronounced in grafted pineals from adult donors although they were the only transplants which were actually denervated. Innervated grafts from all age groups responded to β -stimulation with an increased ^3H NACs production of about the same magnitude as normal pineal glands. ^3H MEL formation in transplants was lower than in the *in situ* pineal glands both in denervated and innervated transplants. It was shown by Bläckström, Olsson and Sjöger (1976) that denervation of transplants caused

MEL (1.9- and 2.3-fold respectively) ($p < 0.05$) but did not alter ^3H -5-HIAA formation (Table 1).

When all non-stimulated transplants from the different donor groups were compiled there was no difference between those that were sympathetically innervated and those that were denervated in the formation of ^3H -labelled NAcS, MEL or 5-HIAA per transplant (Table 1). β -stimulation by KWD 2033 (10^{-6} M) caused a 2.7 times larger increase in ^3H -NAcS production in denervated transplants than in innervated transplants ($p < 0.001$). In the innervated group KWD 2033 increased ^3H -NAcS formation 6.1 times (Table 1). This large ^3H -NAcS increase, induced by KWD 2033, was significantly higher than in the pineal glands of the experimental rats. KWD 2033 (10^{-6} M) also caused a 3.8-fold increase in the formation of ^3H -MEL in denervated transplants ($p < 0.01$). In innervated transplants there was no increase in ^3H -MEL formation following KWD 2033 addition (Table 1).

When innervated and denervated transplants were analyzed with respect to the maturity of the donor animal it was found that the three different fetal groups were approximately equivalent (Fig. 2) while transplants obtained from adult donors were found to increase their ^3H -NAcS formation less in response to β -stimulation. They also had lost much of their ^3H -MEL synthesizing capacity. ^3H -5-HIAA formation in transplants was not affected by denervation or drug treatment (Table 1 and Fig. 2).

Discussion

In the denervated eye chamber pineal transplants a low amplitude variation of NAT enzyme activity persisted (Bäckström, Olson and Seiger 1976). This might have been due to development of denervation supersensitivity of the transplants, which might have responded to circulating catecholamines. The eye chamber transplantation technique also offered a possibility to study such a phenomenon in very immature fetal pineal glands that had not been innervated by the time of operation and where ganglionectomy would have posed technical difficulties. It may thus be possible that these fetal tissues develop an adrenergic receptor supersensitivity without ever having been in contact with their appropriate nerves.

Culture medium content of the 3 ^3H -serotonin metabolites NAcS, MEL and 5-HIAA (5-hydroxyindoleacetic acid) were assayed to obtain an estimate of NAT, HIOMT and monoamine oxidase (MAO) enzyme activities respectively. Determination of ^3H -5-HIAA was considered of importance to ascertain the specific adrenoceptor effect of the agonist and exclude a mere inhibition of serotonin decarboxylation. When this metabolic pathway which alternates with N-acetylation of serotonin, is inhibited a "mass-action" shunt over to N-acetylation has been suggested (Axelrod, Shein and Wurtman 1969; Shein 1971). A newly synthesized β -adrenoceptor agonist KWD 2033 (Carlsson *et al.* 1975) was chosen for the stimulation of pineal adrenoceptor mediated N-acetylation of ^3H -serotonin. KWD 2033 has recently been shown to have maximal efficacy and potency for stimulation of rat pineal gland β -adrenoceptors in organ culture (Bäckström 1976). Metabolite production was measured in the culture medium calculated per pineal and no compensation was made for the differences in amount of tissue between *in situ* pineal glands and transplants.

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an extinction of HIOMT diurnal variation. Thus, eye chamber transplants behaved similarly to *in situ* pineal glands in that they are also dependent on an intact sympathetic innervation for their diurnal HIOMT variation (Axelrod Wurtman and Snyder 1965). From the present experiments it can be seen that 24 h incubation with a directly acting β -adrenoceptor agonist could increase the production of ^3H MEL in denervated transplants but not in innervated transplants, although the latter displayed a dark induced rise in HIOMT activity *in oculo*. The reason for this discrepancy is not readily explained. Nagle, Cardinal and Rowe (1973) found that intraperitoneal injections of noradrenaline caused significant increase in pineal HIOMT activity only in ganglionectomized rats and did not affect enzyme activity in intact animals. They interpreted these findings as a phenomenon of denervation supersensitivity. The present observations on ^3H MEL formation in denervated transplant might be similarly explained. Pineals from adult donors grafted to sympathetically innervated eyes lose some of their capacity to form ^3H MEL in spite of restoration of the sympathetic innervation. It is possible that the capacity of pineal grafts from adult donors to survive as functional eye chamber transplants is less than for fetal tissues.

Innervated and denervated transplants produced ^3H 5-HIAA in equally large amounts as the *in situ* pineal glands. Total MAO activity (measured in homogenates) has been reported to fall 30–50% following denervation of rat pineal glands (Snyder Fischer and Axelrod 1965; Håkansson and Owman 1966; Snyder 1968) mainly through elimination of MAO-A isoenzyme activity (Johnston 1968) which is predominantly contained in the sympathetic nerve terminals in the rat pineal (Goridis and Neff 1971; Neff *et al.* 1974). Serotonin deamination was reported to decrease 70% as compared to normal pineals when measured in homogenates of chronically denervated pineal glands (Goridis and Neff 1971). Any denervation induced decrease in ^3H 5-HIAA formation was not revealed in the present experiment. This might be explained by a change of enzyme affinity for serotonin within the fraction of MAO enzyme remaining despite denervation. It can be noted that Håkansson and Owman (1965) found no decrease in pineal gland MAO activity for 21 days post denervation when ^3H -serotonin was used as substrate for incubations of intact glands. Bläckström and Wetterberg (1973) showed that chronically denervated *in situ* pineals cultured for 24 h in medium containing 0.25 mM ^3H -serotonin, produced as much ^3H 5-HIAA as did normal *in situ* pineal glands in culture.

We conclude that pineal glands transplanted into sympathetically innervated host eyes become innervated regardless of the state of innervation at lack of such at the time of operation. The present experiments lend support to the suggestion that denervated eye chamber pineal transplants might become supersensitive to aminergic stimulation and that this supersensitivity could explain the discreet NAT enzyme alternation between dark and light that was seen in denervated transplants. It is not necessary for a normally sympathetically innervated tissue ever to have been innervated to develop a "denervation supersensitivity to sympathomimetic amines.

Local Reflex in Microcirculation in Human Skeletal Muscle

By

O. HENRIKSEN and P. SEJSEN

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Abstract

HENRIKSEN, O. and P. SEJSEN. Local reflex in microcirculation in human skeletal muscle. *Acta physiol. scand.* 1977 99 19-26.

The effect of venous stasis of 40 mmHg upon blood flow in human skeletal muscle was studied in four normal subjects and in five chronically sympathectomized patients. Blood flow in skeletal muscle was measured by the local ^{133}Xe washout technique. Blood flow decreased about 50 per cent during venous stasis of 40 mmHg. If "passive vascular bed" induced by means of histamine, blood flow decreased only by 16 per cent, indicating that the decrease in blood flow is due to vasoconstrictor response to increase in vascular transmural pressure. The vasoconstrictor response was unaffected by spinal sympathetic blockade, but is blocked in areas infiltrated with lidocaine or with phenolization. The vasoconstrictor response was present in the nonoperated limbs used as control, but abolished in the denervated areas in the five chronically sympathectomized patients. The findings strongly suggest that the vasoconstrictor response in skeletal muscle is due to local nervous mechanism involving adrenergic fibres. Thus local reflex mechanism, most likely sympathetic vasoconstrictor reflex, seems to be present in human skeletal muscle as in collagenase and subcutaneous tissue. This indicates that about 45 per cent of the change in total vascular conductance, less person changes from supine to upright position, is due to this local reflex mechanism operating independently of the central nervous system.

Blood flow in human skeletal muscle decreased about 50 per cent when the leg was placed in dependent position (Ballal et al. 1971; Amery et al. 1973). This vasoconstrictor response to increase in vascular transmural pressure has also been demonstrated in human subcutaneous adipose tissue (Henriksen, Levin Nielsen and Parake 1973) and human cutaneous tissue (Henriksen et al. 1973). The vasoconstrictor response has been shown to be due to local sympathetic reflex mechanism in these tissues (Henriksen and Ahoer 1975; Henriksen 1976 a, b, c, and d; Henriksen and Sejsen 1976 a).

The purpose of the present study was to investigate whether there is a local reflex mechanism in the microcirculation in human skeletal muscle also. If so, the local reflex mechanism might account for a great deal of the total change in vascular conductance during changes from supine to upright position.

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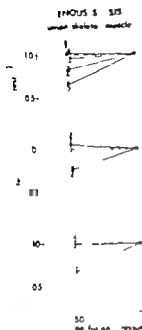


Fig. 1



Fig. 2

Fig. 1. Relative blood flow to human skeletal muscle during venous stasis of 40 mmHg, obtained from normal subjects placed against perfusion pressure head (mmHg). f_{mean} denotes perfusion coefficient obtained during venous stasis, and f_{up} the average perfusion coefficient calculated from the values obtained before and after the test. Control, \circ rats and treated with phentolamine, \bullet areas infiltrated with lidocaine, \bullet areas infiltrated with histamine. Vertical lines with bars denote 1 S.E. Figures denote number of experiments.

Fig. 2. Relative blood flow to human skeletal muscle during venous stasis of 40 mmHg, obtained before and after sympatric blockade induced by epidural anesthesia, placed against perfusion pressure head before blockade, \circ after blockade. Vertical lines with bars denote 1 S.E. Figures denote number of experiments.

The test became negative after the epidural anesthesia had been induced and was still negative after conclusion of the measurements, indicating that the sympathetic fibres had been blocked. Arterial blood pressure remained almost constant during the measuring period. f_{rel} obtained just after the blockade was effective was approximately 50 per cent higher than that obtained just before the blockade. Blood flow decreased 40% before ($p < 0.01$) and 33% ($p < 0.05$) after the blockade during venous stasis of 40 mmHg (Fig. 1). There was no difference in the response ($p > 0.7$).

Lidocaine and phentolamine blocked the vasoconstrictor response to venous stasis as blood flow remained constant corresponding to a decrease in vascular resistance (Fig. 1). When lidocaine (3×10^{-3} mol/l) was applied, relative blood flow \pm S.E. was 0.97 ± 0.05 ($p > 0.6$).

When the tissue was infiltrated with histamine, f_{rel} increased from 0.017 min⁻¹ to 0.56 min⁻¹. During venous stasis, blood flow in these areas decreased 16 per cent ($p < 0.001$) (Fig. 1). This response differed significantly from that obtained in controls ($p < 0.001$).

Methods

The experiments were carried out on four healthy subjects and two chronically sympathectomized patients operated because of manual hyperhidrosis.

Room temperature was about 22°C and remained constant during the investigations. Blood flow in the anterior tibial muscle was estimated by the local ^{133}Xe washout technique (Lassen, Lindbjerg and Munk 1964). The γ -emission was detected by means of NaI(Tl) scintillation detector, collimated to record only from the distal half of the labelled area in order to avoid interference from activity emitted by ^{133}Xe accumulating in fat tissue along the veins. The measurements were started 20 min prior in order to minimize the interference of the injection trauma. A decrease in vascular transmural pressure was induced by venous stasis. A cuff was placed on the thigh of the upper arm, and the ^{133}Xe washout rate constant, k , was measured consecutively: 1) with the cuff deflated (k_{rest}), 2) with the cuff inflated to 40 mmHg, k_{40} , and 3) with the cuff deflated (k_{rest}). Each period of measurement lasted about 3 min, and the count rate was plotted once every 10 s (20°C).

1. Venous stasis

(a) *Central nervous blockade* In order to test the possible contribution by central reflex mechanisms the effect of a total sympathetic blockade upon the response to venous stasis was investigated in one subject. A catheter was placed in the epidural space before the measurements were started. Three experiments were carried out before and 3 after the epidural anaesthesia had been induced by marcaine (Henriksen and Aisner 1975). The sweat test (Dhuner *et al.* 1960) was performed before and after the infusion of marcaine and after the measurements were terminated.

(b) *Local nervous blockade* The effect of local nervous blockade or α -adrenoceptor blockade upon the response to venous stasis was studied by injecting 1 ml of phentolamine (10 mg/ml) and 1 ml of lidocaine with 1% vasoconstrictant (20 mg/ml 3×10^{-4} mol/l) mixed with 0.1 ml ^{133}Xe in an isotonic saline into the anterior tibial muscle. The experiments were started 30 min later with the subject placed in a supine position.

(c) *Blockade of the microcirculation* A passive vascular bed was induced by injecting 0.1 ml of histamine as the base (1 mg/ml) mixed with ^{133}Xe into the anterior tibial muscle. The activity was recorded every 5 s, and each period of measurement lasted only 1.5 min.

(d) *Measurement of venous pressure* Venous pressure was measured directly on the dorsum of the hand during venous stasis in one subject placed in a supine position.

II. Chronic sympathetic denervation

(1) The experiments were performed on the brachioradial muscle of both arms of a unilaterally sympathectomized patient operated about 9 years before. The hand on the side of operation was completely dry while there was still manual hyperhidrosis on the other side (control side). The effect of venous stasis upon muscle blood flow was studied in both arms.

(2) In a bilaterally sympathectomized patient, studied two years postoperatively, measurement were performed on the brachioradial muscle, and the anterior tibial muscle was used as control.

Calculation and statistics

Mean perfusion coefficient, f , can be calculated from the Kety-equation $f = k/100$ (ml/100 g \cdot min) (Kety 1951), where k denotes the washout rate constant, min^{-1} , and λ the muscular tissue blood partition coefficient (ml/g) (0.7 ml/g) (Lassen *et al.* 1964).

Relative blood flow $f_{\text{rest}}/f_{\text{ven}}$ equals relative ^{133}Xe washout rate constant $k_{\text{rest}}/k_{\text{ven}}$ if the tissue to blood partition coefficient λ is assumed to be equal in the two situations. k_{rest} and f_{rest} here denote average value of the washout rate constants (min^{-1}) and the perfusion coefficient (ml/100 g \cdot min) obtained just before and after the test respectively.

k_{rest} and k_{ven} were compared by means of the Student's *t*-test for paired samples. The calculated relative blood flow values obtained during stasis and under the different conditions were compared by means of the randomization test for unpaired samples. Level of significance was chosen as 0.05.

Results

Blood flow decreased 30 per cent during venous stasis of 40 mmHg in all 4 subjects (Figs 1 and 2).

During venous stasis the relative distribution volume of ^{133}Xe will increase due to accumulation of blood in the distended veins, resulting in a decrease in the washout rate constant. Direct measurements of blood pressure in the superficial veins revealed that venous pressure increased close to the cuff pressure within 1 min, indicating that the major part of the increase in venous volume has occurred within this time. ^{133}Xe solubility in blood and muscle tissue has been found to be 2.5 and 1.6 respectively relative to water (Cooms 1961). Assuming that the vascular volume after 1 min of venous stasis of 40 mmHg has increased maximally from 5 to 9% of the tissue volume, the increase in relative volume of distribution would be about 3.6%. Thus the washout rate constants obtained after 2 min of stasis are then underestimated by less than 4%.

Increase in filtration of fluid out of the vascular system during venous stasis might also interfere. Assuming that the capillary filtration capacity in skeletal muscle is 0.01 ml/100 g min mmHg (Cobbold *et al* 1963) and that the increase in mean capillary pressure is 25 mmHg during venous stasis of 40 mmHg, the error introduced by 1 min of venous stasis could be less than 1%. Even when the tissue is infiltrated with histamine, the error is insignificant, because capillary filtration capacity probably increases maximally four times (Cobbold *et al* 1963), indicating that the error would be less than 4%.

Vasomotor response

Blood flow in skeletal muscle decreased 30% during venous stasis of 40 mmHg. In a "passive vascular bed" induced by histamine, blood flow decreased by only 16% during venous stasis of 40 mmHg. Reference blood flow was approximately 39 and 2 ml/100 g min with and without histamine, respectively. With histamine the calculated vascular resistance is similar to that obtained after work to exhaustion during 10 min of ischemia, indicating that the vascular tone was almost abolished. This difference in response between the normal and the "passive" vascular bed indicates that the decrease in blood flow during venous stasis of 40 mmHg is due to arteriole constriction in the normal area. This is compatible with the observed increase in vascular resistance in skeletal muscle to increase in transmural pressure induced by postural changes (Balldin *et al* 1971; Amery *et al* 1973).

The response was not affected by central nervous blockade, but was blocked by lidocaine and phenolamine. In these cases, blood flow remained constant during venous stasis of 40 mmHg, corresponding to a decrease in vascular resistance of 45%. The response was present in the control limbs but abolished in the denervated limbs in the chronically sympathectomized patients.

The effect of sympathectomy upon the vasoconstrictor response might be due to secondary affection of vascular smooth muscle reactivity following degeneration of sympathetic fibres. However, initiation and propagation of rhythmic activity in smooth muscles in small subcutaneous arteries in dogs were not affected (Johansson and Bohr 1966). Also, reactivity of cat portal vein to acetylcholine was not diminished by chronic sympathectomy (Johansson *et al* 1964). In humans, autoregulation of blood flow in subcutaneous tissue was present years after sympathectomy (Hennriksen 1976 b).

The blocking effect of lidocaine and phenolamine might be due to direct action on the

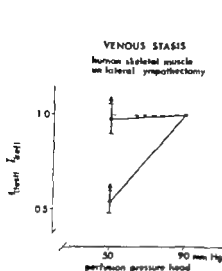


Fig. 3

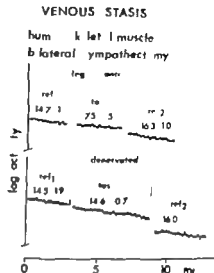


Fig. 4

Fig. 3 Relative blood flow in human skeletal muscle during venous stasis of 40 mmHg, obtained from the non-operated side (control) and the operated side (denervated) in a unilaterally sympathectomized patient about nine years after the operation. \circ = control side, \bullet = denervated side. Vertical lines with bars denote 1 S.E. Figures denote number of expts.

Fig. 4 ^{133}Xe washout curves obtained before, during and after venous stasis of 40 mmHg in a bilaterally sympathectomized patient studied about 2 years postoperatively. Upper curves obtained from the non-operated leg used as control, lower curves are obtained from a denervated forearm. Figures above the curves denote the washout rate constants ($k \cdot 10^3 \text{ (min}^{-1})$).

II Chronic sympathetic denervation

Blood flow decreased 45% ($p < 0.01$) in response to venous stasis on the control side but remained constant ($p > 0.8$) on the denervated side in the unilaterally sympathectomized patient (Fig. 3). The difference in response was significant ($p < 0.02$). In the other patient relative blood flow during venous stasis, $f_{\text{stasis}}/f_{\text{ref}} \pm \text{S.E.}$ was 0.88 ± 0.05 on the denervated forearm ($p > 0.05$) and 0.54 ± 0.04 ($p < 0.01$) on the leg used as a control. An example of the ^{133}Xe washout curves obtained in this patient is shown in Fig. 4.

Discussion

Washout rate

The ^{133}Xe washout rate found 20 min after the injection is influenced by accumulation of the tracer in fat cells lining the vessels (Tonnesen and Sejrsen 1967) and "veno-arterial" shunting of ^{133}Xe by diffusion of inert gas in skeletal muscle (Sejrsen and Tonnesen 1972). Influence of activity emitted from ^{133}Xe accumulated in these fat cells was minimized by collimating the detectors to record only from the distal part of the labelled area. Due to the "veno-arterial" shunting by diffusion, blood flow calculated from the ^{133}Xe washout curve obtained 20 min after the injection at resting conditions corresponds to a value lying between 40 and 80% of the directly measured venous outflow (Tonnesen and Sejrsen 1970).

The findings in the present study are similar to those obtained in human cutaneous tissue and human subcutaneous adipose tissue (Henriksen and Sejrøen 1976 a, Henriksen and Alkner 1975, Henriksen 1976 a, b, c, d). This means that blood flow in cutaneous tissue, subcutaneous adipose tissue, and skeletal muscle decreases about 50 % due to this local reflex mechanism, when venous transmural pressure is elevated about 25 mmHg or more.

Plethysmography

Blood flow measured by venous plethysmography might be influenced by the reflex. With a cuff pressure of 40 mmHg it took between 40 and 60 s for the venous pressure to increase to the threshold level of the reflex, 25 mmHg, indicating that the reflex is not elicited during venous occlusion plethysmography for measurements of blood flow at low venous pressures.

When the method is used for measurement of capillary filtration capacity (Mellander Öberg and Odehman 1964), possible involvement of the reflex should be considered.

Haemodynamic implications of the reflex

1) Postural changes from supine to upright position caused an average decrease in cardiac output of 1.7 l/min (Hanson, Torkelson and Levy 1968), corresponding to a decrease in total vascular conductance of about 22 %. Assuming that venous transmural pressure increases more than 25 mmHg in tissues corresponding to 50 % of the b.wt. and that the average decrease in blood flow is 2 ml/100 g · min in these tissues, the local reflex mechanism could account for a decrease in vascular conductance of about 10 %, which is 45 % of the total change. This means that about 45 % of the postural changes in vascular tonus is due to this local reflex, occurring independently of the central nervous system. The remaining 55 % are probably due to central reflex mechanisms elicited from baroreceptors located in the large arteries, central veins, and heart (Roddie and Shepherd 1957, Benzer *et al.* 1970, Rowell, Wyse and Brongehusen 1973).

2) Autoregulation of blood flow (i.e. maintenance of constant blood flow during changes in arterial perfusion pressure head) became evident when the reflex was blocked, indicating that autoregulation is due to intrinsic mechanisms.

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vascular smooth muscle cells affecting myogenic reactivity Lidocaine ($3 \cdot 10^{-4}$ M) did not affect myogenic activity in the rat portal vein, but blocked impulse transmission in sympathetic fibres effectively (Johansson and Ljung 1967). In this concentration, lidocaine blocked the vasoconstrictor response effectively. In two experiments phentolamine, $5 \cdot 10^{-6}$ mol/l, was applied. Even in this low concentration phentolamine blocked the vasoconstrictor response to venous stasis induced 15 min after the injection. Four control experiments with injection of isotonic saline performed in order to test the effect of the injection trauma indicated that this trauma was without effect 15 min after the injection. Phentolamine ($5 \cdot 10^{-6}$ M) did not affect myogenic activity in the rat portal vein (Johansson and Mellander 1975).

The effect of lidocaine and phentolamine is, therefore, most likely due to a blockade of sympathetic adrenergic vasoconstrictor fibres indicating that the vasoconstrictor response is due to a local nervous mechanism.

Folkow and Öberg (1961) observed that venous stasis of 10 mmHg induced a decrease in blood flow in skeletal muscle in reserpinized cats, indicating that vascular reactivity is still present in more or less complete catecholamine depleted tissue. The duration of the periods of measurement was less than 40 s. Jones and Berne (1964) observed a transient decrease in blood flow in the thigh muscle of dog during venous stasis of 20 mmHg, lasting about 40 s, whereafter blood flow almost returned to the level before the period of stasis. Lundvall and Mellander (1976) made similar observations in skeletal muscle of cat during external negative pressure of 40 mmHg. These observations suggest an intrinsic vascular response to increase in vascular transmural pressure.

In humans a steady decrease in blood flow was observed during the venous stasis. Days after sympathectomy this response was abolished, indicating that the vasoconstrictor response to venous stasis in humans rather is neurogenic than an intrinsic vascular response.

The findings in the reserpinized cats (Folkow and Öberg 1961) is in agreement with the observation that autoregulation in subcutaneous tissue was retained years after the sympathectomy (Henriksen 1976 b). This observation does not exclude an overlying neuro-reinforcing mechanism.

When increase in venous transmural pressure during lowering of the limb by 40 cm was prevented by letting the subject tip his foot, the vasoconstrictor response was blocked in subcutaneous tissue as vascular resistance increased only by 48% corresponding to the increase in arterial perfusion pressure head (autoregulation) (Henriksen and Sejrsen 1976 b). The abolishment of the vasoconstrictor response during exercise might be due to liberation of metabolites in subcutaneous tissue affecting myogenic activity. However, elevation of venous transmural pressure during exercise by inducing venous stasis elicited an increase in vascular resistance of the same magnitude as in the lowered, resting leg. In patients with venous insufficiency exercise did not prevent the increase in venous transmural pressure and did not abolish the vasoconstrictor response. Thus the abolishment of the vasoconstrictor response to lowering during exercise, is probably due to prevention of increase in venous transmural pressure, indicating that the vasoconstrictor response depends upon an impulse transmission from veins to arterioles. This furthermore strongly suggests that the vasoconstrictor response is due to a local nervous mechanism, probably a sympathetic axon reflex.

Efflux of 5-Hydroxytryptamine from Synaptosomes of Rat Cerebral Cortex

By

SVANTE B. ROSS and DIANA KJELDER

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Abstract

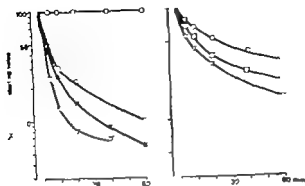
ROSS, S. B. and KJELDER, D. *Efflux of 5-hydroxytryptamine from synaptosomes of rat cerebral cortex.* Acta physiol scand. 1977 99 27-36.

The efflux of ³H-5-HT from crude synaptosome preparation of the cerebral cortex of reserpinized rats is examined. The synaptosomes were loaded with ³H-5-HT by pre-incubation of the homogenate in presence of pargiline and desipramine in order to inhibit degradation of 5-HT and uptake into noradrenergic neurons. The synaptosomes are collected by centrifugation, washed and resuspended in 0.25 M sucrose. No spontaneous efflux of 5-HT was detectable at 0°C but marked efflux was observed at 27°C and in the presence of 4-chloroamphetamine, low external Na⁺ concentration, ouabain and the depolarizing agent veratrine markedly accelerated the initial (5 min) efflux. Inhibitors of the neuronal 5-HT uptake, *p*-chloroamphetamine, H 102/09 and A 23189 antagonized the 5-HT efflux evoked by these means, whereas desipramine, such a poor inhibitor of the 5-HT uptake, had only slight effect on the 5-HT efflux. It is suggested that 5-HT can be actively transported out from the synaptosomes by the reversed 5-HT uptake mechanism.

En mots: 5-Hydroxytryptamine efflux, 4-chloroamphetamine, chloroamphetamine, H 102/09, A 23189

The physiological importance of the neuronal membrane uptake mechanisms for the biogenic monoamines is generally recognized and accepted. Due to the hydrophilic nature of these amines they slowly pass the neurone membranes by diffusion and active uptake mechanisms are therefore necessary for a rapid transport of these amines through the membranes. It is also a general accepted view that the indirectly acting sympathomimetic amines exert their action by releasing the biogenic monoamines from intraneuronal binding sites (Trendelenburg 1972). From pharmacological observations it has been concluded that this release is different from that produced by nerve stimulation (Furchtgott *et al.* 1963). Recently Thoa *et al.* (1975) found that the release of noradrenaline by the sympathomimetic amines in contrast to that of nerve stimulation in the isolated vas deferens was not joined by release of the enzyme dopamine β -hydroxylase. If the release of the biogenic amines does not occur with an exocytosis mechanism the problem remains how the released amines pass the neurone membranes outwardly since the same barrier exists in both directions. Until recently this problem has been overlooked, although it was suggested as possible that

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1 Efflux of ^3H -5-HT from cerebral cortical synaptosomes of reserpined rats. Synaptosomes were loaded with ^3H -5-HT by incubation of cortical homogenates with ^3H -5-HT (10^{-6} M) in presence of 0.1 M parvalbumin and 10^{-4} M desipramine as described in Method. The washed crude synaptosome preparation (100 μl) containing 27 ± 3 (4) pmol ^3H -5-HT/g original tissue was incubated in 2.0 ml Krebs-Henseleit buffer or modified sucrose containing buffer low in Na (26 mM). The efflux of ^3H -5-HT determined by measuring the radioactivity in the synaptosomal pellets after various times of incubation and expressed as per cent of the starting value. Each value is the mean of 4 determinations, all \pm SE of 4 or less. A incubation at 0°C (broken lines) and 37°C (solid lines). B incubation at 37°C in the presence of 4-chloroamphetamine (5×10^{-6} M) (open triangles) and H 102 09 (10^{-6} M) (open circles).

Results

Efflux of ^3H -5-HT from rat brain synaptosomes at various temperatures

The time course of the efflux of ^3H -5-HT from the cortical synaptosome preparation was studied at three different temperatures and at various incubation conditions (Fig. 1). At 0°C almost no efflux was detectable during the time examined. At 37°C the efflux of 5-HT from the synaptosomes was rapid and addition of 4-chloroamphetamine (5×10^{-6} M) to the incubation medium caused a further increase in the efflux (Fig. 1A). At 27°C the rate of efflux was slower than at 37°C (Fig. 1B). The data obtained gave a Q_{10} value of 2.5 for the initial effluxes observed at these two temperatures. In an incubation medium with low Na (substituted with sucrose) the efflux was more rapid than in the normal incubation medium. Addition of ouabain (5×10^{-6} M) and simultaneous omission of K in the incubation medium also accelerated the efflux of ^3H -5-HT (Table II). The depolarizing agent veratridine (5×10^{-6} M) caused pronounced increase in the 5-HT efflux at 37°C (70.9 ± 1.6 pmol/g.5 min, $4 \text{ P } 0.001$) but no effect was observed at 0°C. The effect by veratridine was antagonized by tetrodotoxin (10^{-6} M) (69 ± 5 inhibition, $n = 4$, $\text{P } 0.01$) but had no effect on the 5-HT efflux evoked by low external Na (0^* inhibition). Omission of Ca in the incubation medium, did not influence on the veratridine evoked efflux (71.3 ± 1.1 pmol/g.5 min, $4 \text{ P } 0.05$).

The rapid spontaneous efflux of 5-HT at 37°C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their content into the medium. This possibility was examined by testing the ability of the synaptosomes to accumulate ^3H -5-HT. The synaptosomes were prepared exactly as for the efflux experiments

cocaine may antagonize the indirectly acting amines by inhibiting the outward passage of noradrenaline through the neurone membrane (Ross *et al* 1968, Ross and Renyi 1971). Experimental observations have shown that the efflux of noradrenaline from rat brain slices and slices of atria from reserpinized rabbits is an active energy dependent transport which is inhibited by desipramine and cocaine (Blazkowski and Bogdanski 1972, Paton 1973). Using bretylium as a model compound for the membranous noradrenaline transport mechanism in noradrenergic neurones of the rat vas deferens we have observed that the compound is transported actively outwardly as well as inwardly by a desipramine sensitive mechanism (Ross 1976a, Ross and Kelder 1976). In the present study we have examined if a similar outward transport mechanism exists for 5-HT in central 5-HT neurones.

Materials and Methods

Crude synaptosomes from reserpinized male Sprague-Dawley rats (150-180 g) were prepared from the cerebral cortex according to the method of Snyder and Coyle (1969). Cerebral cortical slices were homogenized in 10 ml of ice-chilled 0.5 M sucrose in a Potter Elvehjem glass homogenizer. The homogenate was centrifuged at 800 g for 10 min. The supernatant (8 ml) was incubated for 15 min at 17°C with 15.4 ml of Krebs-Henseleit buffer pH 7.4 containing 4×10^{-4} M pargyline, 1×10^{-4} M desipramine (to inhibit uptake of 5-HT in noradrenergic neurones), 1×10^{-4} M ascorbic acid, 1×10^{-4} M EDTA and 5.4×10^{-4} M glucose. 5-HT was then added giving a final concentration of 1×10^{-6} M and the incubation was continued for 20 min. The incubation mixture was centrifuged for 70 min at 16 000 g in a Sorvall RC3 centrifuge. The tubes and pellets were washed 3 times with 5 ml of 0.25 M sucrose solution. The pellets were re-homogenized in 8 ml of 0.5 M sucrose and centrifuged at 16 000 g for 70 min. The pellet obtained were homogenized in 8 ml of 0.25 M sucrose and this synaptosome suspension was used for the efflux experiments. The uptake of 5-HT in synaptosomes was determined as described previously (Ross and Renyi 1975).

The efflux of 5-HT from the synaptosomes was determined under the following experimental conditions. A aliquot of 100 μ l of the synaptosome suspension was added to 1.9 ml of Krebs buffer or modified buffer containing 2.4×10^{-4} M pargyline, 1×10^{-4} M ascorbic acid, 1×10^{-4} M EDTA and, if present, the test compound in a PVC centrifuge tube. The incubation was performed in a water bath at 37°C otherwise as stated. Immediately after incubation the tubes were centrifuged at 16 000 g for 30 min. The pellet and the tubes were washed with 5 ml of cold saline solution and the tubes were rinsed from water drops with tissue paper. The pellets were dissolved in 1.0 ml of Soluene-130 (Packard) at room temperature for one hour. Ten ml of the scintillation liquid were added and the radioactivity measured in a liquid scintillation spectrometer. Standards of 5-HT and blanks were run simultaneously. The amount of radioactivity in the pellet is expressed in pmol 5-HT/g original tissue. Control experiments in which 5-HT was separated from deaminated metabolites by thin layer chromatography (Ross and Renyi 1969), showed that after 0 and 60 min incubation the amount of 5-HT in the pellet and in the medium was 95% of the total radioactivity. Reserpine (5 mg/kg *p*) was injected 18 h prior to the experiments.

The final incubation medium (Krebs-Henseleit buffer) contained 117 mM NaCl, 5.4 mM KCl, 0.6 mM MgSO₄, 2.6 mM CaCl₂, 1.1 mM NaH₂PO₄ and 25 mM NaHCO₃ and, as required, treated with O₂ containing 6.5% of CO₂ giving a pH of 7.4. In some experiments this buffer was modified by substituting NaCl with 0.5 M sucrose or 117 mM LiCl giving a Na⁺ concentration of 26 mM.

Compounds

5-Hydroxytryptamine-HCl creatinine sulphate (10.7 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Pargyline was gift from Abbott Lab. Inc. and chlorimipramine and desipramine from J. R. Geigy A.G. 11- β -(2,3-(4-bromophenyl)-N,N-dimethyl-3-(3-pyridyl)allyl)ammonium chloride and A-3189 (3-aminopropanoic acid 1-(4-chlorophenyl)-1-methyl-2-propylester hydrochloride) were synthesized in the Astra laboratories.

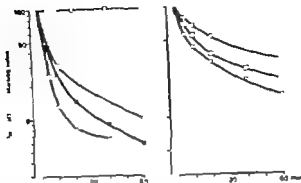


Fig. 1. Efflux of ^3H -5-HT from cerebral cortical synaptosomes of reserpinated rats. Synaptosomes were loaded with ^3H -5-HT by incubation of cortical homogenate with ^3H -5-HT (1×10^{-6} M) in presence of 10^{-6} M ganglione and 3×10^{-6} M desipramine described in Method. The washed crude synaptosome preparation (100 μl) containing 227 \pm 31 μmol ^3H -5-HT/g wet weight was incubated in 0.1 M Krebs-Henseleit's buffer or modified sucrose containing buffer low in Na (76 mM). The efflux of ^3H -5-HT is determined by analyzing the radioactivity in the extracellular perfusate after known times of incubation and expressed as per cent of the starting value. Each line is the mean of 4 determinations, all \pm S.E. of 4 or less. A: Incubation at 0°C (broken lines) and 37°C (solid lines). B: Incubation at 37°C. () Normal medium; (●) medium low in Na; (Δ) 4-chloroamphetamine, 5×10^{-6} M; (○) ^3H -5-HT, 10^{-6} M.

Results

Efflux of ^3H -5-HT from rat brain synaptosomes at various temperatures

The time course of the efflux of ^3H -5-HT from the cortical synaptosome preparation was studied at three different temperatures and at various incubation conditions (Fig. 1). At 0°C almost no efflux was detectable during the time examined. At 37°C the efflux of 5-HT from the synaptosomes was rapid and addition of 4-chloroamphetamine (5×10^{-6} M) to the incubation medium caused further increase in the efflux (Fig. 1A). At 37°C the rate of efflux was slower than at 37°C (Fig. 1B). The data obtained gave a Q_{10} value of 2.5 for the initial efflux observed at these two temperatures. In an incubation medium with low Na (substituted with sucrose) the efflux was more rapid than in the normal incubation medium. Addition of ouabain (5×10^{-6} M) and simultaneous omission of K^+ in the incubation fluid also accelerated the efflux of ^3H -5-HT (Table II). The depolarizing agent tetrathiodine (5×10^{-6} M) caused pronounced increase in the 5-HT efflux at 37°C (70.9 ± 1.6 pmol/g/5 min, $P < 0.001$) but no effect was observed at 0°C. The effect by tetrathiodine was antagonized by tetrodotoxin (1×10^{-6} M) (69 ± 5 inhibition, $P < 0.01$) but had no effect on the 5-HT efflux evoked by low external Na (0 inhibition). Omission of Ca^{2+} in the incubation medium, did not influence on the tetrathiodine evoked efflux (71.3 ± 1.1 pmol/g/5 min, $n = 4$, $P > 0.05$).

The rapid spontaneous efflux of 5-HT at 37°C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their content into the medium. This possibility was examined by testing the ability of the synaptosomes to accommodate ^3H -5-HT. The synaptosomes were prepared exactly as for the efflux experiment.

cocaine may antagonize the indirectly acting amines by inhibiting the outward passage of noradrenaline through the neurone membrane (Ross *et al* 1968, Ross and Renyi 1971). Experimental observations have shown that the efflux of noradrenaline from rat heart slices and slices of atria from reserpinized rabbits is an active energy dependent transport which is inhibited by desipramine and cocaine (Blazkowski and Bogdanski 1972, Pike 1973). Using bretyllium as a model compound for the membranal noradrenaline transport mechanism in noradrenergic neurones of the rat vas deferens we have observed that the compound is transported actively outwardly as well as inwardly by a desipramine sensitive mechanism (Ross 1976a, Ross and Kelder 1976). In the present study we have examined if a similar outward transport mechanism exists for 5-HT in central 5-HT neurones.

Materials and Methods

Crude synaptosomes from reserpinized male Sprague-Dawley rats (150-160 g) were prepared from the cerebral cortex according to the method of Snyder and Coyle (1969). Cerebral cortical slices were homogenized in 10 ml of medium containing 0.3 M sucrose in a Potter E Rheym glass homogenizer. The homogenate was centrifuged at 800 g for 10 min. The supernatant (8 ml) was incubated for 15 min at 37°C with 13.4 ml of Krebs-Henseleit buffer pH 7.4 containing 4×10^{-4} M pargyline, 1×10^{-4} M desipramine (1 inhibitory pot. of 5-HT in noradrenergic neurones), 1×10^{-4} M ascorbic acid, 1×10^{-4} M EDTA and 5.4×10^{-4} M glucose. 5-HT was then added giving a final concentration of 1×10^{-6} M and the incubation was continued for 20 min. The incubation mixture was centrifuged for 10 min at 16,000 g in a Sorvall RC3 centrifuge. The tubes and pellets were washed 3 times with 5 ml of 0.25 M sucrose solution. The pellets were homogenized in 8 ml of 0.3 M sucrose and centrifuged at 16,000 g for 10 min. The pellet obtained was homogenized in 8 ml of 0.3 M sucrose and this synaptosome suspension was used for the efflux experiments. The uptake of [3 H]-5-HT in synaptosomes was determined as described previously (Ross and Renyi 1975).

The efflux of [3 H]-5-HT from the synaptosomes was determined under the following experimental conditions. A aliquot of 100 μ l of the synaptosome suspension was added to 1.9 ml of Krebs buffer or a modified buffer containing 4×10^{-4} M pargyline, 1×10^{-4} M ascorbic acid, 1×10^{-4} M EDTA and, if present, the test compound. PVC centrifuge tubes. The incubation was performed in a water bath at 37°C if otherwise is not stated. Immediately after incubation the tubes were centrifuged at 16,000 g for 10 min. The pellets and the tubes were washed with 5 ml of cold saline solution and the tubes were rinsed from water drips with tissue paper. The pellets were dissolved in 10 ml of Soluene-150 (Packard) at room temperature for one hour. Ten ml of the scintillation liquid were added and the radioactivity measured in a liquid scintillation spectrometer. Standards of [3 H]-5-HT and blank were run simultaneously. The amount of radioactivity in the pellets is expressed in pmol [3 H]-5-HT/g original tissue. Control experiments in which 5-HT was separated from deaminated metabolites by thin layer chromatography (Ross and Renyi 1969), showed that after 0 and 60 min incubation the amount of [3 H]-5-HT in the pellet and in the medium was 95% of the total radioactivity. Risperine (5 mg/kg p.o.) was injected 18 h prior to the experiment.

The normal incubation medium (Krebs-Henseleit buffer) contained 117 mM NaCl, 5.4 mM KCl, 0.6 mM MgSO₄, 0.6 mM CaCl₂, 1.1 mM NaH₂PO₄ and 5 mM NaHCO₃ and was equilibrated with O₂ containing 6.5% of CO₂ gas. pH of 7.4. In some experiments this buffer was modified by replacing NaCl with 0.3 M sucrose or 117 mM LiCl giving a concentration of 76 mM.

Compounds

5-Hydroxytryptamine- 3 H(G) creatinine sulphate (10.7 C mmol) was purchased from the Radiochemical Centre, Amersham, England. Pargyline was gift from Abbott Lab. Inc. and chlorimipramine, desipramine from J. R. Geigy A.G. [3 H]-10,09 [(Z)-1-(4-bromophenyl)-N,N-dimethyl-1-(4-methylamino)-2,3-dihydrochloride] and A-3189 (1-aminopropanoic acid 1-(4-chlorophenyl)-2-methyl-1-propylester hydrochloride) were synthesized in the Astra laboratories.

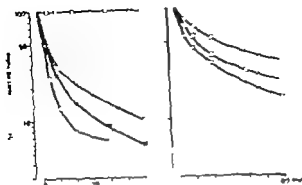


Fig. 1. Efflux of ^3H -5-HT from cortical cortical synaptosomes of reserpinized rat. Synaptosomes were loaded with ^3H -5-HT by incubation of cortical homogenate with ^3H -5-HT (10^{-6} M) in presence of 10^{-4} M paralytic and 10^{-4} M desipramine as described in Methods. The shed crude synaptosome suspension (100 ml) containing 2.7×10^6 (A) pmol ^3H -5-HT g original tissue incubated in 0.1 M HEPES buffer or modified sucrose containing buffer low in Na (26 mM). The efflux of ^3H -5-HT is determined by analyzing by radioactivity in the synaptosomal pellets after known times of incubation and expressed in per cent of the starting value. Each value the mean of 4 determinations, all ± 4 S.E. of 4 or less. A incubation 37°C (broken lines) and 37°C (solid lines). B incubation at 27°C (medium lines), medium low in Na (—) 4-Chloroamphetamine at 5×10^{-6} M (---) 11.10. 09 10^{-6} M (—).

Results

Efflux of ^3H -5-HT from rat brain synaptosomes at various temperatures

The time course of the efflux of ^3H -5-HT from the cortical synaptosome preparation was studied at three different temperatures and at various incubation conditions (Fig. 1). At 0°C almost no efflux was detectable during the time examined. At 37°C the efflux of 5-HT from the synaptosomes was rapid and addition of 4-chloroamphetamine (5×10^{-6} M) to the incubation medium caused a further increase in the efflux (Fig. 1 A). At 27°C the rate of efflux was slower than at 37°C (Fig. 1 B). The data obtained give a Q_{10} value of ~ 5 for the initial effluxes observed at these two temperatures. In an incubation medium with low Na (substituted with sucrose) the efflux was more rapid than in the normal incubation medium. Addition of ouabain (5×10^{-6} M) and simultaneous omission of K⁺ in the incubation fluid also accelerated the efflux of ^3H -5-HT (Table II). The depolarizing agent estradiol (5×10^{-6} M) caused a pronounced increase in the 5-HT efflux at 37°C (70.9 ± 1.6 pmol/g/5 min ± 4 P < 0.001) but no effect was observed at 0°C. The effect by estradiol was antagonized by tetrodotoxin (1×10^{-6} M) (69 ± 5 inhibition ± 4 P < 0.01) but had no effect on the 5-HT efflux evoked by low external Na (0% inhibition). Omission of Ca^{2+} in the incubation medium, did not influence on the estradiol evoked efflux (71.3 ± 1.1 pmol/g/5 min ± 4 P < 0.05).

The rapid spontaneous efflux of 5-HT at 37°C may suggest that the synaptosomes are not stable under these incubation conditions but are broken and release their contents into the medium. This possibility was examined by testing the ability of the synaptosomes to accumulate ^3H -5-HT. The synaptosomes were prepared exactly as for the efflux experiments

TABLE I Inhibition of the efflux of ^3H 5-HT from synaptosomes evoked by 4-chloroamphetamine (5×10^{-6} M), low external Na^+ (26 mM NaCl replaced by 250 mM sucrose) and by veratridine (3×10^{-6} M). The cortical synaptosomes were loaded with ^3H 5-HT (1×10^{-6} M) in presence of 2.4×10^{-4} M pargyline and 3×10^{-6} M desipramine for 20 min. The efflux of ^3H 5-HT was determined by incubating 100 μl of the washed suspension in 1.0 ml Krebs buffer or modified buffer for 5 min at 37°C . The difference in the amount of radioactivity in the pellet in absence and presence of the efflux inducer was taken as the evoked efflux. The inhibition of the evoked efflux was calculated in per cent. Each value is the mean \pm S.E. of 4 determinations.

Compound	Conc. M	Inhibition		
		4-Chloro- amphetamine	Low Na	Veratridine
H 102/09	2.5×10^{-6}	14 ± 2	—	—
	2.5×10^{-5}	48 ± 2	61 ± 6	59 ± 6
A 23189	3.4×10^{-6}	48 ± 4	60 ± 14	69 ± 5
	2.9×10^{-5}	—	36 ± 11	43 ± 3
Chlorimipramine	2.9×10^{-6}	79 ± 3	89 ± 5	63 ± 3
	3.0×10^{-5}	12 ± 3	70 ± 5	19 ± 4
Desipramine	3.0×10^{-6}	—	9 ± 3	40 ± 3
	2.9×10^{-5}	—	8 ± 4	82 ± 3
Millicaine	2.9×10^{-5}	—	—	—

$0.05 > P > 0.01$

$0.01 > P > 0.001$

$P < 0.001$ (Student's *t*-test)

with the exception that ^3H 5-HT was omitted in the pre-incubation of the homogenate. The uptake of ^3H 5-HT for a period of 5 min was examined at times corresponding to the start of the efflux experiment and after 60 min incubation at 37°C . The accumulation decreased from 64.2 ± 9.0 ($n = 5$) to 16.8 ± 1.8 ($n = 5$) pmol/g tissue/min incubation i.e. the uptake after 60 min incubation was only 26% of that at zero time. Thus, the partially purified synaptosomes had lost a large part of their capacity to accumulate ^3H 5-HT by the incubation.

Effect of uptake inhibitors on the efflux of ^3H 5-HT

The effect of H 102/09, a selective inhibitor of the membranal 5-HT uptake (Ross et al. 1976) on the efflux of ^3H 5-HT from the synaptosomes at 37°C was examined (Fig. 1A). H 102/09 itself caused an increase of the spontaneous efflux, which was more marked after prolonged incubation. However, it significantly inhibited the initial increase of the efflux produced by 4-chloroamphetamine, by low external Na and by veratridine (Table I). Chlorimipramine (Shaskan and Snyder 1970) and A 23189 (unpublished observations) are two other potent inhibitors of the 5-HT uptake. As shown in Table I these compounds inhibited the efflux of ^3H 5-HT induced by the various means examined. Desipramine which is a potent inhibitor of the NA uptake but a rather poor inhibitor of the 5-HT uptake (Ross and Renvi 1975) had much less effect in inhibiting the 5-HT efflux. At the higher concentration of desipramine examined the effect on the veratridine induced efflux was larger than on that evoked by low external Na. The same discrimination between the efflux evoked by veratridine and low external Na was observed for the local anesthetic agent millicaine (Table I).

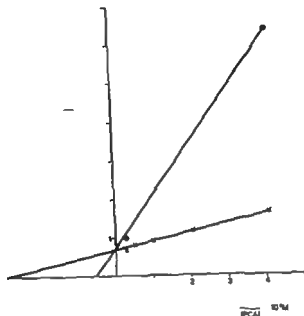


Fig. 2 Double reciprocal plot of the increase of the ^3H -5-HT effect caused by 4-chloroamphetamine (PCA). Synaptosomes were prepared and loaded with ^3H -5-HT as described in the legend of Fig. 1. The incubation time was 5 min. The velocity of efflux (v) was determined as the difference of the efflux at 37°C and 0°C and was expressed in pmol/g/min. The concentration of H 102/09 (O) was $2.5 \cdot 10^{-7}$ M. Each value is the mean of 4 determinations.

Kinetics of the antagonism of H 102/09 on the 4-chloroamphetamine induced increase of 5-HT efflux

The kinetics of the antagonism of H 102/09 on the increased ^3H 5-HT efflux produced by 4-chloroamphetamine was examined according to the method of Lloerweaver and Bark (1934). The increased initial (5 min) efflux caused by 5 different concentrations of 4-chloroamphetamine ($2.4 \cdot 10^{-6}$ M– $3.9 \cdot 10^{-5}$ M) were determined in absence or presence of H 102/09 ($2.5 \cdot 10^{-7}$ M). The result of an experiment is shown in Fig. 2. H 102/09 inhibited competitively the effect of 4-chloroamphetamine. The apparent K_m for 4-chloroamphetamine in increasing the efflux was $4.6 \cdot 10^{-6}$ (mean of 2 determinations, $5.4 \cdot 10^{-6}$ M and $3.7 \cdot 10^{-6}$ M). The inhibitor constant (K_i) for H 102/09 was $1.1 \cdot 10^{-6}$ M (mean of $1.5 \cdot 10^{-6}$ M and $6.1 \cdot 10^{-7}$ M). Both these values are close to the corresponding K_i values for the inhibition of the 5-HT uptake in the same synaptosome preparation being $1.2 \cdot 10^{-6}$ M for 4-chloroamphetamine and $1.8 \cdot 10^{-6}$ M for H 102/09 (Fig. 3). The apparent K_m value for 5-HT was in these experiments $8 \cdot 10^{-6}$ M.

Effect of ouabain on the increase in efflux produced by 4-chloroamphetamine

It was of interest to examine, if ouabain antagonized the increase in efflux produced by 4-chloroamphetamine, since if so, it should indicate that 4-chloroamphetamine is actively transported by a Na⁺ dependent mechanism into the synaptosomes before it releases 5-HT

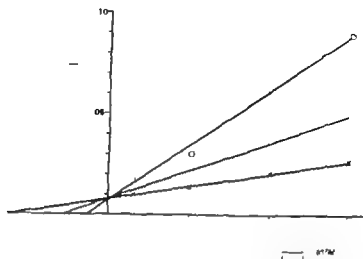


Fig. 3. Double reciprocal plot of the uptake of H³-5-HT by cerebral cortical synaptosomes from reserpine-treated rats. The incubation time was 5 min. The uptake velocity (v) was determined as the difference (37°C and 0°C) and was expressed in pmol/g/min. The concentration of H³ (10^{-10} M) was $2.5 \cdot 10^{-1}$ M and that of 4-chloroamphetamine (○) $5 \cdot 10^{-6}$ M. Each value is the mean of 4 determinations.

intraneuronally. The synaptosomes were simultaneously incubated with ouabain in a medium free of K⁺ and 4-chloroamphetamine at low concentration ($1 \cdot 10^{-6}$ M) in order to be within an adequate concentration range for the potential uptake (Table II). The effect of this combination was larger than that of 4-chloroamphetamine and ouabain alone, which indicates an additive effect.

Kinetic determination of the Na⁺ dependence of the 5-HT transport

Since both influx and efflux of 5-HT in synaptosomes are influenced by Na⁺ (Bogdanski *et al.* 1968) it was of interest to examine kinetically the nature of the Na⁺ dependence. This was best performed by working with the uptake system, since the 5-HT concentrations can then be properly controlled. In this experiment we used non-reserpine-treated rats and the crude cortical homogenate obtained after low speed centrifugation (800 g). The determination of the uptake of H³-5-HT ($3 \cdot 10^{-6}$ M– $2 \cdot 10^{-5}$ M) by the synaptosomes in the homogenate was performed in normal buffer and in buffer low in Na⁺ (26 mM) by replacing NaCl with 117 mM LiCl in order to keep the Cl⁻ concentration unchanged. The double reciprocal plots (Fig. 4) show that the affinity of 5-HT for the uptake sites was decreased by the low

TABLE II. The combined effect of ouabain ($5 \cdot 10^{-6}$ M), no external K⁺ and 4-chloroamphetamine ($1 \cdot 10^{-6}$ M) on the efflux of H³-5-HT from cerebral cortical synaptosomes of the rat. The incubation was performed for 5 min. Each value is mean \pm S.E. of 8 determinations.

		Δ Efflux pmol/g min \pm S.E.	P
1	Ouabain + 0 K ⁺	50 ± 0.3	
2	4-Chloroamphetamine	102 ± 0.3	
3	4-Chloroamphetamine + ouabain + 0 K ⁺	12.8 ± 0.1	0.001 (1)

Student's t-test

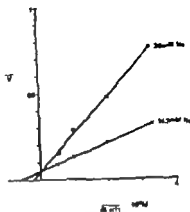


Fig. 4. Double reciprocal plot of the uptake of 5-HT in synaptosomes of rat cerebral cortex (non-transported) at two different Na⁺ concentrations. The incubation time was 5 min. The uptake velocity (V) as determined as described in the legend of Fig. 3.

external Na⁺ concentration, whereas V_{max} was unchanged. The apparent K_m value for normal Na⁺ concentration ($1.7 \cdot 10^{-3}$ M) was in this experiment twice higher than that obtained with the more purified synaptosomes obtained from reserpinized rats.

Discussion

The marked spontaneous efflux of ³H-5-HT from the cerebral cortical synaptosomes observed in this study is in accordance with the findings by Bogdanaki *et al.* (1968). Since it was temperature dependent it is probably due to metabolic processes. Bogdanaki *et al.* (1968) found that the labelled 5-HT decreased more rapidly than the endogenous 5-HT in the synaptosomes and proposed that dilution of the labelled amine with newly synthesized 5-HT was one reason for the decline in the labelled 5-HT. No attempt to test this hypothesis with an inhibitor of the 5-HT synthesis has been performed. Another possible mechanism is that the synaptosomes are unstable under the incubation conditions employed, are broken and release their contents out to the medium. The loss of a large part of the capacity of the synaptosomes to accumulate 5-HT after 60 min incubation at 37°C indicates that a part of the synaptosomes deteriorate in some way. This may at least partially contribute to the rapid spontaneous efflux of 5-HT observed at this temperature.

In spite of this spontaneous efflux of 5-HT very constant and reliable values were obtained when determining the initial 5 min efflux evoked by 4-chloroamphetamine, low external Na⁺ ouabain and veratridine. Since this evoked 5-HT efflux was temperature dependent and was antagonized by uptake inhibitors it is probably an active outward transport of 5-HT. The observation that H 102/09 which is a selective inhibitor of the neuronal 5-HT uptake (Rom *et al.* 1976), accelerated the 5-HT efflux but antagonized the efflux evoked by amox means appears somewhat conflicting. The acceleration of the efflux is understood, if it is supposed that a part of 5-HT released is taken up again into the synaptosomes by the membranous transport mechanism. The finding that the effect of H 102/09 increased with time is in accordance with this interpretation, since the medium concentration of 5-HT increased and accordingly the uptake velocity. The antagonism of the evoked efflux is,

on the other hand, understood, if the transport of 5-HT under these experimental conditions occurs with the reversed uptake mechanism and this efflux accordingly inhibited by uptake inhibitors.

There are several observations supporting the hypothesis that the membranal 5-HT transport mechanism can be reversed. The Na^+ dependence of the influx and efflux of 5-HT (Bogdanski *et al* 1968) indicates this possibility since several other Na^+ dependent transport mechanisms are known to be reversible (Schultz and Curran 1970). Na^+ influences the transports either by increasing the affinity of the solute for the uptake sites or by increasing the velocity of the transport. Observation in the present study indicates that the affinity of 5-HT is increased by Na^+ which is in variance with the transport systems for noradrenaline (Sugrue and Shore 1971) and dopamine (Holz and Coyle 1974) in which the velocity of the transport but not the affinity appears to be increased. The explanation of the effect of 5-HT by low external Na^+ may accordingly be due to an equalization of the affinities on the two sides of the neurone membrane, which because of the much larger 5-HT concentration inside the synaptosomes results in the outward transport. Ouabain by inhibiting the Na^+ pump increases the Na^+ concentration in the synaptosomes and thereby the affinity of the 5-HT for the carrier sites on the inside of the membrane. Veratridine increases the Na^+ permeability of excitable membranes (Ulbricht 1969) with the same result as with ouabain but with a more rapid onset of action. The finding that the local anesthetic agent millicaine discriminated between the 5-HT efflux induced by veratridine and low external Na^+ indicates that the membrane stabilizing effect of the test compounds was not involved in the inhibition of the 5-HT efflux mechanism. Thus, the effect by millicaine on the veratridine evoked efflux could be explained by the antagonism of the depolarizing effect whereas the corresponding effect by the uptake inhibitors was probably due to the inhibition of the 5-HT transport. In accordance with this view a comparatively high concentration of desipramine, which is a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975), had larger effect on the veratridine evoked efflux, reflecting the membrane stabilizing action at this concentration. Indeed inhibition of the release of neurotransmitters induced by veratridine in synaptosomal preparations may become a valuable method for testing drugs on the membrane stabilizing action under identical or similar *in vitro* conditions as used in other assays. However the test compound must not inhibit the active outward transport of the transmitter examined.

The efflux induced by 4-chloroamphetamine can also be explained by the hypothesis of the reversed transport mechanism. This amine has high affinity for 5-HT neurones (Pletscher *et al* 1964; Fuller *et al* 1965). Since the vesicular storage mechanism of 5-HT was destroyed by reserpine, 4-chloroamphetamine, which because of its large lipophilicity readily passes membrane barriers, probably released 5-HT bound to some extravascular binding sites in the nerve endings. The rise in the concentration of the soluble fraction of 5-HT in the synaptosomes results in increase in the outward transport of 5-HT since the rate of the transport is directly related to the concentration of 5-HT within the ranges of the transport capacity. The inhibition by the 5-HT uptake inhibitors of the efflux evoked by 4-chloroamphetamine, low external Na^+ and high internal Na^+ at the same concentration ranges support this hypothesis. It can be argued that the uptake inhibitors antagonized

an active transport of 4-chloroamphetamine into the synaptosomes and thereby preventing the intraneuronal release of H 5-HT. However the finding that ouabain did not inhibit the effect of 4-chloroamphetamine but instead added its own effect to that of 4-chloroamphetamine indicates that 4-chloroamphetamine was not transported by the membranal 5-HT transfer system. Experiments with purified synaptosomes have also shown that H 4-chloroamphetamine has high affinity for the synaptosomes but no active, Na⁺ dependent accumulation was observed (Ross 1976 b).

The inhibition by 4-chloroamphetamine of the 5-HT uptake in the synaptosomes may not in fact be due to a real inhibition of the uptake but due to its 5-HT releasing effect. If the hypothesis of a reversible transport mechanism is correct the influx of 5-HT should equilibrate with the efflux at a much lower ratio between the inner and outer concentration of 5-HT than under normal condition, if the extravesicular binding sites are occupied by 4-chloroamphetamine, which results in an elevated concentration of the free concentration of 5-HT. This hypothetical effect of 4-chloroamphetamine should accordingly be very similar to an inhibition of the uptake although the primary attack is not located to the transfer sites. This suggestion is in accordance with the observation that 4-chloroamphetamine was more or at least as potent in increasing the efflux than in inhibiting the uptake of 5-HT. According to the kinetic experiments H 102/09 inhibited competitively the increased efflux produced by 4-chloroamphetamine. Since the release by 4-chloroamphetamine of intraneuronally bound ³H 5-HT was probably directly related to the concentration of 4-chloroamphetamine the observed competitive nature of the effect of H 102/09 is not contradictory to the hypothesis that H 102/09 inhibited the outward transport of 5-HT. The almost identical K_i values for H 102/09 in inhibiting the 5-HT uptake and the 4-chloroamphetamine induced 5-HT efflux further support the hypothesis of an identical mechanism for the uptake of 5-HT and the efflux of 5-HT evoked by 4-chloroamphetamine.

Alternative explanations of the data are possible. For instance, it can not be excluded that the influx and efflux are two separate mechanisms and that the inhibitors of the 5-HT uptake also antagonized the efflux mechanism due to similar structure requirements for both mechanisms.

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Preferential Uptake of ^3C - α Aminoisobutyric Acid into Mouse Uterine Tissue during Early Pregnancy

By

INGER LINDQVIST, OVE NILSSON* and GUNNAR RÖNQVIST

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Abstract

LINDQVIST I, O NILSSON and G RÖNQVIST. Preferential uptake of ^3C - α -aminoisobutyric acid into mouse uterine tissue during early pregnancy. *Acta physiol. scand.* 1977 99 37-41.

The uptake of ^3C -AIB into uterine tissue and some other organs was studied during early implantation. Mice in experimentally delayed implantation were given estrogen and ^3C AIB at different time intervals. Mice in delayed implantation given only ^3C AIB displayed low transport rate into the uterine tissue, comparable with that for the diaphragm muscle. However, if the estrogen injection preceded that of the ^3C -AIB by 4 to 8 h the transport capacity increased by a factor of at least 4 times, while the uptake for the diaphragm muscle remained low. Under such conditions ^3C AIB-accumulation into uterine tissue was favored for at least 4 h of *in vivo* incubation with the labeled amino acid.

The rate of amino acid transport appears to be closely correlated to the rate of protein synthesis of both normal and malignant tissues (Riggs 1964, Griffiths 1967). Glucocorticoids, which are catabolic hormones, work antagonistically to the transport of amino acids into skeletal muscle (Riggs 1964, Christensen 1975), while insulin and growth hormone, for example, which are both anabolic hormones and promote protein synthesis accelerate amino acid transport into several tissues (Riggs 1964). Also estrogens promote growth of particular tissues (Noall *et al.* 1957, Noall and Allen 1961) and consequently McCorquodale and Mueller (1958) observed an increased activity of amino acid-activating enzymes in rat uterus already 3 h after treatment with estrogen. An early "induced protein" after exposure of rat uterus to estradiol, has recently been detected among the soluble proteins (Petrequin, Robel and Baeklen 1975).

The capacity to transport amino acids is probably also higher since the level of free amino acid is higher in the more rapidly growing tissues (Noall *et al.* 1958). This is tested by using α -aminoisobutyric acid, AIB, as an amino acid substrate since this amino acid is not metabolized. As expected, Noall *et al.* (1958) found a 3-fold increase in the uterine accumulation of AIB upon administration of estradiol, and Riggs and Pan (1972) observed an increased

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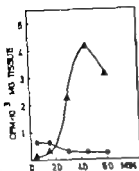


Fig. 1

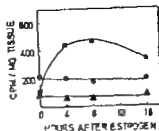


Fig. 2

Fig. 1 ^{14}C AIB concentration in blood and recovery in uterus at different times after intravenous administration. \bigcirc — \bigcirc blood, \triangle — \triangle uterus.

Fig. 2 Recovery of ^{14}C -AIB in blood (\bigcirc — \bigcirc), diaphragm muscle (\triangle — \triangle) and uterine tissue (\square — \square) at different stages of implantation initiated by estrogen. Stand. dev. \pm max \pm 40 cpm.

most of the other 3 groups were given an injection of estrogen, and ^{14}C AIB was administered 20 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of ^{14}C -AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The *in vivo* uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The *in vivo* uptake by the uterine tissue, on the contrary displayed a characteristic profile with a low uptake of ^{14}C AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by estrogen and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of ^{14}C -AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different *in vivo* incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals in experimentally delayed implantation were used. The animals were sacrificed 1/3, 4, 16, 24 and 48 h after the injection of ^{14}C AIB (Fig. 3).

Although the blood concentration of the labeled amino acid was declining already at 20 min after the i.v. administration (cf. Fig. 1) and remained low throughout the expt., the ^{14}C -AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 20 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of ^{14}C AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed a somewhat longer accumulation time and higher uptake than the diaphragm muscle. The uptake of ^{14}C AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process into brain tissue *in vivo* might be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with a relatively slow transport rate for AIB (Oldendorf

in vitro transport of AIB into the estrogen primed uterus. Other studies also indicate an influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al.* 1968, Walters *et al.* 1975).

Since early pregnancy specifically the time of blastocyst implantation, is associated with an increase in the concentration of estrogen we questioned whether the capacity of AIB transport is increased as well. If so this might have implications for the nutrition of the blastocyst at trophoblast attachment and invasion. Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some organs of mice, conditioned for experimentally delayed implantation (Humphrey 1967) and secondly to examine whether the capacity of AIB transport *in vitro* into the uterus and some other organs increased after the initiation of implantation by estrogen.

Materials and methods

¹⁴C- α -aminoisobutyric acid (1 ¹⁴C AIB), Protosol and Aquasol were purchased from NEN Chemical GmbH Dreieichenbain, W. Germany and α -aminoisobutyric acid from Calbiochem, San Diego, USA. Progesterone was purchased from Ikapharm, Ramat-Gan, Israel and estradiol from AB Leo, Helsingborg, Sweden. All chemicals used were analytical grade. Radioactivity was measured in a Nucleo Chicago UNIBOX II Liquid Scintillation Counter.

Adult white female mice (N.M.R.I. Naval Medical Res. Inst., Bethesda, USA) were mated and the day the vaginal plug was seen was recorded as day 1. They were spayed on day 3 and given the first dose of 1 mg of progesterone in 0.04 ml peanut oil subcutaneously the same day. The animals were kept in delayed implantation by daily injections of progesterone. Implantation was initiated by a subcutaneous injection of 0.1 g estradiol 17 β in 0.1 ml propylene glycol. Generally 4.25 μ Ci ¹⁴C AIB in 0.1 ml of a 40 mOsm AIB solution, made isotonic with NaCl, was given intrathecally in the tail to each animal. Blood samples were drawn from the cut tail and directly transferred to the liquid scintillation bottles. Tissue samples were washed once in ice-cold Krebs-Ringer bicarbonate buffer freshly bubbled with a gas mixture of 93.5 O₂ and 6.5 CO₂ to give a pH of 7.4 and lightly blotted on tissue paper before being transferred to the bottles. The animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred to weighed liquid scintillation counting bottles, weighed and dissolved overnight in 1 ml Protosol at 50°C. The solutions were mixed with 30 μ l conc. acetic acid (1 murexure ¹⁴C/g sensitivity) and 10 ml of Aquasol. Samples were then counted and counts/g corrected for self absorption.

Results

The clearance of ¹⁴C AIB was established by determining the time at which the amino acid concentration curve of the blood crossed that of the urine. The labeled amino acid was intravenously administered to the animals which were then sacrificed at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of ¹⁴C AIB after 20 min. The curve profiles of both blood and urine were quite similar in 4 different expts. with animals in delayed implantation as well as with estrogen-treated animals. It was therefore convenient to sacrifice the animals 10 min after the intravenous administration of ¹⁴C-AIB during the next series of expts.

The distribution of ¹⁴C AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice. In experimentally delayed implantation, animals of one group (controls) were given only ¹⁴C AIB 20 min before

During activation for implantation the first 8 h are a period when system A amino acids are supplied from the blood into the uterine tissue at a high degree. Perhaps this accumulation of amino acids in the uterine tissue can be exploited also by the developing blastocyst. If so, AIB should accumulate also in the blastocysts. This possibility is presently being tested.

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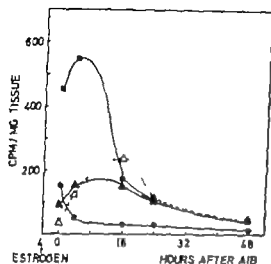


Fig. 3 Retention time of ^{14}C AIB in blood (●—●), diaphragm muscle (—), brain (Δ—Δ) and uterine tissue (■—■). ^{14}C AIB was administered 4 h after estrogen initiation of implantation. Stand. dev. = max \pm 50 cpm.

Discussion

Mice in delayed implantation which in addition to progesterone had received estrogen or 8 h before being given ^{14}C AIB i.v. displayed a high uterine uptake compared with mice not having received estrogen. Furthermore this uptake was preferential for the uterine tissue compared with some other tissues. Since the ^{14}C AIB transport was considerably low when only ^{14}C AIB was given pretreatment *in vivo* with estrogen is necessary for the enhanced ^{14}C AIB-accumulation. This is in agreement with the findings of Riggs *et al.* (1968) for the *in vitro* uptake of AIB in immature rat uterus after *in vitro* preincubation with estradiol. Since the estrogen effect is not momentaneous it seems probable that the hormone might induce a *de novo* synthesis of AIB-carriers in the plasma membranes of uterine tissue. Such a proposal can be further supported by the determination of the V_{max} parameter and Riggs *et al.* (1968) have shown that in their experimental system V_{max} increased while K_m remained unchanged by the treatment with estradiol.

AIB has been shown to be transported mainly by the route defined as the system A by Oxender and Christensen (1963). Several other amino acids as alanine, methionine and nor-leucine are mainly transported by this route in different cells. Characteristical features for this system are the Na^+ -dependency and transport against a concentration gradient. This means necessarily that the exit rate by this system is slower than the entrance rate (Christensen and Handlogten 1968) and that the carrier operates asymmetrically. By this system it is therefore possible to augment the free intracellular amino acid pool while an exchange system can only influence the qualitative composition of the intracellular amino acid pool without enhancing the net uptake. Such an asymmetry has been shown to exist in slices of newborn rat kidney cortex while absent in adult cortex (Webber 1968, Webber and Cairns 1968).

The strict Na^+ -dependence of the AIB-uptake into uterine tissue can naturally not be demonstrated by *in vitro* expts. However Riggs *et al.* (1972) have shown that the uptake of AIB into the estrogen primed rat uterus *in vitro* increased regularly with increasing Na^+ amounts in the incubation buffer. Thus we have reasons to believe that the system A is

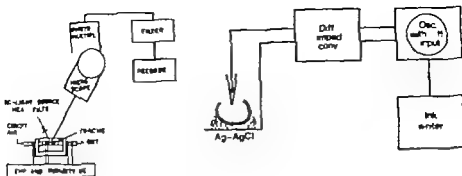


Fig. 1. Block diagram of the experimental equipment for recording mucociliary wave frequency (left) and intracellular activity (right).

lower temperatures is due to physical properties of the secretion layer and not to the intracellular "pacemaker" mechanism. However this conclusion was drawn from recordings of the intracellular and extracellular activities in separate specimens. Simultaneous recordings from the same area would be even more valuable for theoretical and practical purposes. The aim of the present investigation has therefore been 1) to devise a method for the simultaneous recording of the intracellular electrical activity and the extracellular mucociliary wave frequency in the same area on tracheal specimens and 2) to compare the frequencies of the electrical discharge and the mucociliary movements at different temperature levels within the range of 20–40°C.

Methods and material

Rabbits weighing 1.5–2.0 kg have been used as experimental animals. To avoid undesired pharmacological side effects they have been sedated by blow on their heads. After careful dissection of the trachea 3–4 cm specimen has been removed and put into an experimental chamber in which temperature and relative humidity can be regulated and recorded.

Recording of the mucociliary activity

The recording technique has already been extensively described (Mørkild *et al.* 1974). Shortly: cold light beam is directed against the tracheal ciliated mucosa membrane through skin as the upwards directed light membrane. Variations of the light intensity of the mucosal reflex are picked up by microscope (Fig. 1). A photomultiplier (EMI 9524 B) attached to one of the oculars converts the light intensity variations to electrical signals which after amplification and filtering (Kroebe Hite type 3350) are recorded by an inkwriter (Elema Mingograph 34).

Recording of the intracellular electrical activity

The technique of recording is a modification of the method originally described by Håkanson and Torrenius in 1964 and has in detail been described by Torrenius *et al.* (1975). In the actual investigation further improvement has been introduced by exchanging the formerly used transimpedance converter to low frequency impedance converter with resistance of 1 000 MΩ and an amplification of 11 (Fig. 1).

Experimental procedures

After the tracheal specimen has been placed into the experimental chamber, the capillary microelectrode is very slowly pushed downwards through the mucus into the cell layer in the center of the light reflex. The oscilloscope (first in the DC position). When potential change of 15 to 40 mV indicates that ciliated

Intra and Extracellular Activity of Ciliated Cells

By

D. HUBERMAN, C. H. HÅKANSSON, U. MERCKE and N. G. TOREMÄLM

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Abstract

HUBERMAN D., C. H. HÅKANSSON, U. MERCKE and N. G. TOREMÄLM: *Intra- and extracellular activity of ciliated cells*. Acta physiol. scand. 1977 99 42-47.

A method permitting simultaneous recording *in vivo* of the intracellular and extracellular (= mucociliary) activity of respiratory ciliated mucous membrane is presented. Using this method the influence of different temperature levels (20–30°–40°C) upon the tracheal mucous membrane of 15 rabbits has been investigated. It is shown: 1) that the extracellular (= mucociliary) activity increases with rising temperature whereas the intracellular activity is unaffected and remains constant, indicating a temperature influence upon the respiratory tract mucus rather than a change of the intracellular pace-maker mechanism; 2) that the restraining effect of the mucus decreases with rising temperatures but is still 10% of the intracellular frequency at body temperature; 3) that the presented method should be useful for future investigations concerning the effect of supposition and pharmacological substances upon respiratory mucous membranes.

Key words. Trachea, mucous membranes, ciliated cells, mucociliary activity, electrophysiological activity.

The mechanical defence mechanism of the respiratory mucous membrane is constituted of two factors—the propulsive movements of the cilia and the more or less restricting effect of the covering layer of mucus. The term mucociliary activity has therefore been found more adequate than the previously used designation, “the ciliary beat frequency” (Mercke *et al.* 1974a). It has hitherto not been possible to separate the rhythm and frequency of intrinsic ciliary activity from that of the extrinsic mucociliary activity observed through a microscope, but such a functional difference should be of great practical importance for experimental and diagnostic purposes. However, this is not possible with the use of extracellular recording methods only. A combination of intra- and extracellular measurements is necessary.

The intracellular electrical activity in beating ciliated cells was first recorded in vertebrates by Håkansson and Toremalm in 1966. In recent experimental studies we found that the frequency of intracellular action potentials was about the same at 20–30° and 40°C during *in vitro* conditions while the extracellular movements of the cilia decreased considerably with decreasing temperatures (Toremalm *et al.* 1975). This means that the frequency changes

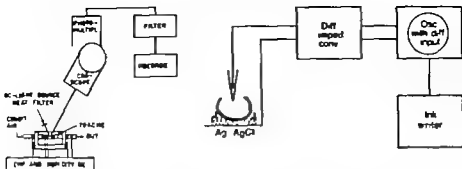


Fig 1 Block diagram of the experimental apparatus for recording muscular activity (left) and neuronal activity (right).

lower temperatures is due to physical properties of the secretion layer and not to the intracellular "pace-maker" mechanism. However this conclusion was drawn from recordings of the intracellular and extracellular activities in separate specimens. Simultaneous recordings from the same area would be even more valuable for theoretical and practical purposes. The aim of the present investigation has therefore been 1) to devise a method for the simultaneous recording of the intracellular electrical activity and the extracellular mucociliary wave frequency in the same area on tracheal specimens and 2) to compare the frequencies of the electrical discharge and the mucociliary movements at different temperature levels within the range of 20–40°C.

Methods and material

3-40 cm specimen has been removed and put into an experimental chamber in which temperature and micro-humidity can be regulated and recorded.

Recording of the masticatory activity

The recording technique has already been extensively described (Marcks *et al.* 1974). Shortly cold light being directed against the tracheal ciliated sensory membrane through slit in the upwards directed pericardial membrane. Variations of the light intensity of the mucosal reflex are picked up by a microscop (Fig. 1). A photomultiplier (EMI 9224 B) attached to one of the oculars converts the light intensity variations to electrical signals, which after amplification and filtering (Kroebe Hite type 3550) are recorded by an inkwriter (Elema, Mergograph 34).

Recording of the intracellular electrical activity

The technique of recording is a modification of the method originally described by Häkansson and Torsellin in 1966 and has as detailed been described by Torsellin *et al.* (1975). In the actual investigation further improvement has been introduced by exchanging the formerly used transformer-coupled impedance converter to a low frequency impedance converter with resistance of 1 000 M Ω and an amplification of 11 (Fig. 1).

Experimental procedure

After the tracheal specimen has been placed into the experimental chamber, the capillary microelectrode is very slowly pushed down as air is drawn through the mucus into the cell layer in the center of the light reflex. The oscilloscope then in the DC position. When potential change of -15 to -40 mV indicates that critical

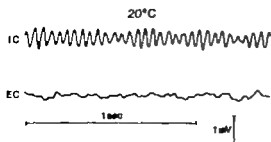


Fig. 2. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary wave pattern (EC) at 20°C.

cell membrane has been penetrated, the oscilloscope is changed in the AC position and the above-described intracellular recording is commenced. The microscope is then adjusted so that variations of the light reflections exactly corresponding to the area of intracellular recording may be picked up and recorded.

The simultaneous recording of intracellular electrical and extracellular mechanical activities from the very small mucosal area has been performed at 3 different temperature levels (20, 30 and 40°C) and at an environmental air humidity above 90%. The trachea from 5 rabbits have been investigated at each temperature level.

Results

At 20°C (Fig. 2) the intracellular potential recordings show a fairly even rhythm and a frequency of 20 cycles/s whereas the extracellular recordings are irregular with a low frequency of about 12 waves/s (Table I).

At 30°C (Fig. 3) the intracellular frequency does not change in contrast to the extracellular activity which has increased. The difference in the intra and extracellular activities is

TABLE I

Temp. (°C)	Intracellular activity (oscillations/s)	Extracellular (mucociliary) activity (waves/s)
20	20.5	9.6
	21.5	13.1
	21.0	17.1
	21.7	10.4
	21.3	12.7
	M: 21.2	M: 12.6
30	21.3	16.7
	21.5	17.4
	23.1	18.0
	21.4	17.6
	20.8	16.3
	M: 21.6	M: 17.2
40	22.1	18.8
	22.0	19.5
	21.5	17.7
	21.2	18.8
	21.5	17.4
	M: 21.5	M: 18.4

Fig. 3. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary wave pattern (EC) at 30°C

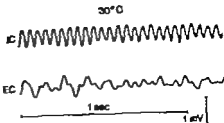
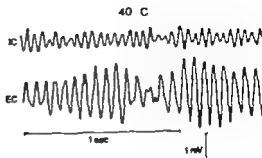


Fig. 4. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary wave pattern (EC) at 40°C.



markedly reduced (20 cycles and 17 waves/s respectively Table I). At 40°C (Fig. 4) this difference is even more reduced, now being 20 cycles and 18 waves/s respectively (Table I).

The frequency/temperature relationship concerning the intracellular as well as the extracellular recordings is illustrated in Fig. 5. The plotted values are the mean frequencies of all 5 rabbit specimens (each temperature level (Table I). From this figure it is clearly evident that the intracellular activity is mainly unaffected by temperature changes in the interval 20°C to 40°C. However the extracellular or mucociliary activity increases almost linearly with increasing temperature in the same interval.

Discussion

A comparative study of the intracellular electrical activity which initiates the movements of the cilia covering the surface of the respiratory tract, and the extracellular manifestations

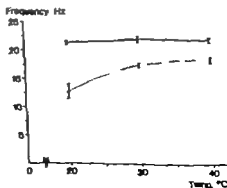


Fig. 5. The frequency/temperature relationship regarding intracellular (broken line) and extracellular (dotted line) activities simultaneously measured. Each plotted value is the average of 5 measurements.

of this activity is presented in this investigation. The experimental model is a combination of two previously described methods (Fig. 1) dealing with one of these activities at a time (Mercke *et al.* 1974 a, Toremalm *et al.* 1975). It has been possible to make simultaneous recordings of the intracellular electrical activity in one ciliated cell and the extracellular mechanical activity from a small area covering about 10 ciliated cells during standardized conditions regarding temperature and humidity of the surrounding air.

This investigation has been limited to a comparison of the two mentioned activities at three different temperature levels. Typical recordings at 20°, 30° and 40°C are demonstrated in Fig. 2, 3 and 4 respectively. The entire results regarding all tracheal specimens are shown in Table I and are also graphically illustrated in Fig. 5.

The relative constancy of the intracellular oscillations from 20 to 40°C is a physiologically interesting phenomenon. In fact this may have two explanations: 1. The rhythm of the "pace-maker" is relatively insensitive to temperature changes. 2. The frequency may be constant but the rising time of the potential fluctuation can be shorter and the rise more rapid at higher temperatures. This may result in an increasing amplitude of the intracellular recording. Such an explanation cannot be deduced from Fig. 2, 3 and 4 since the recordings at 20, 30 and 40°C are obtained from different tracheal specimens. To be able to answer the hypothesis further expts. have to be done where one tracheal specimen is investigated at the different temperatures.

The extracellular mucociliary activity on the other hand is strongly influenced by temperature changes. This has been described more in detail in two previous papers (Mercke *et al.* 1974 b, Mercke 1974). From the present simultaneous recordings frequency differences between the intra and extracellular activities increase by decreasing temperature levels below 40°C (Fig. 5). Ordinary body temperature therefore seems to be optimal for the cilia to work in. In other words the mechanical restriction of the secretion layer is minimal at this temperature level. However even at optimal temperature conditions there is a difference of about 2 cycles/s between the internal and external activities. It means that the secretion layer normally has a decelerating effect upon the movements of cilia. The consequences of the present results are (1) that the method makes it possible to separate the intracellular ciliary activity (the "pace maker" effect) from the retarding effect of the mucus layer surrounding the cilia, and (2) that this inhibitory factor reduces the frequency by about 10% at and above body temperature.

This knowledge seems to be valuable for future experimental investigations in which the model may be used e.g. for air pollution tests and control of pharmacological substances for local treatment of the respiratory tract. From the results a hypothetical conclusion may be drawn implying that it must be easier to stimulate the mucociliary defence mechanism by changing the physical properties of the mucus than to try to accelerate the intracellular activity of the ciliated cells. However this hypothesis need to be proven by a series of further expts. which are now in progress.

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The Effect of Teeth Amputations on the Choline Acetyltransferase Activity of Rat Submaxillary Glands

By

JÖRGEN EKSTRÖM

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Abstract

Ekström J. The effect of teeth amputations on the choline acetyltransferase activity of rat submaxillary glands. *Acta physiol. scand.* 1977 99 48-52.

The choline acetyltransferase activity in parasympathetically decentralized glands was unaffected by repeated teeth amputations over a period of 2 weeks, while after the same period of time the treatment caused the enzyme activity to increase in innervated glands. It appears that the enzyme in the postganglionic nerve is for its activity dependent on an intact connection with the central nervous system. The increase in enzyme activity is attributed to an enhanced reflex stimulation of the glands from pulpal receptors.

Key words: Choline acetyltransferase activity, salivary glands, impulse flow

When the parasympathetic postganglionic neurones of the salivary glands lose their connection with the central nervous system by a section of the preganglionic nerve supply their capacity to synthesize acetylcholine has been found to diminish, as judged from the fall in the activity of choline acetyltransferase (Nordenfelt 1964, Ekström and Holmberg 1972). Based on this observation Nordenfelt (1964, 1965) suggested that the activity of the acetylcholine forming enzyme might be influenced by the intensity of the impulse traffic in the neurones, as to the adrenergic neurones a similar relationship between the activity of catecholamine synthesizing enzymes and impulse traffic has since been considered (Zetler and Pellegrino de Iraldi 1966, Molinoff and Axelrod 1971, Thoenen 1974). The idea put forward by Nordenfelt is favoured by the outcome of various types of experiments on salivary glands, where the degree to which the glands were activated from the central nervous system was either decreased or increased over a long time period (see Ekström 1975). In parotid glands of rats the choline acetyltransferase activity declined when the animals were kept on a liquid diet, while it increased when the diet was dry and bulky or when the mouth and the throat of the animals were made dry by atropinization or salivary duct ligations. The increase in the choline acetyltransferase activity in the submaxillary glands of the rat after repeated teeth amputations reported by Ohlin and Perac (1967) seems also to be a finding, which would fit into the pattern outlined above: the enzyme activity is increased as a conse-

the enhanced stimulation of the glands reflexly elicited by the irritation of palpal receptors. However these authors reported that in the parasympathetically decentralized glands the choline acetyltransferase activity had increased after the amputations. This might suggest that some other factor than the impulse traffic is responsible for the increased enzyme activity seen after teeth amputations.

In the present study the effect of teeth amputations on the choline acetyltransferase activity was compared in decentralized and innervated submaxillary glands.

Methods

50 male rats of Sprague-Dawley strain bred at this Institute were used. They were at the start of the expts. 4-6 months old. The diet used was of grain-like consistency prepared daily by mixing powder made of standard pelleted diet with water, so as to make a mass for the rats with amputated teeth to eat, the rats serving as controls were also given this diet. The diet and water were given *ad libitum*. Both at the start and at the end of the experimental period the body weights of the rats were determined.

In rats highly sensitized, both lower and upper incisors are cut to the gingival margin with bone cutting forceps every other day over a period of either 14 or 21 days. The rats serving as controls were also exposed to ether for a few minutes at the same occasion.

In one type of expt. the chorda-lingual nerve was bilaterally cut under dissecting microscope in 18 rats, and a few cm. of the nerve was removed. The wound was then sutured. 9 of these rats with amputations started 7 days later and continued over a time period of 14 days, the other 9 rats were litter mates to be amputated later and served as controls. In another type of expt. rats with an intact innervation of their salivary glands were exposed to the amputations. In first series, the incisors of 9 rats were amputated over a period of 14 days, in second series 7 rats were amputated over a period of 21 days, 9 and 7 unoperated litter mates to these rats were amputated and served as controls.

At the end of the experimental period the rats were killed by inhalation of ether and the submaxillary glands were removed (the sublingual glands being separated from the submaxillaries), washed in saline, cleaned, pressed gently between gauze pads and weighed. In the rats studied over the period of 3 weeks the sublingual glands were also removed and treated in the same way as the submaxillaries. Both at end dry weights were determined, the latter expressed as acetone dried powder.

The glands were then prepared for the analyses of their activity of choline acetyltransferase using the method derived by Hebb (see Nordenskjöld 1963, 1965). Acetone dried powder was made of the 2 pooled submaxillary glands of one rat, as to the sublingual glands each pool consisted of the glands of 7 rats. The powder of the glands was made up in glycine-saline in a concentration of 30 mg./ml. Of the tissue extract 0.2 ml was incubated at 37°C for 60 min. Experimental preparation and control preparation were incubated simultaneously and the assay for acetylcholine was made on the same emulsified frog cortex. The choline acetyltransferase activity is expressed as μg acetylcholine chloride formed per h per pooled glands (total activity) and as μg acetylcholine chloride formed per h per g acetone powder (concentration).

Student's *t*-test as used, paired comparisons were made between the experimental rat and its control litter mate. *P* values of less than 0.05 were considered significant.

Results

Body weights. At the start of the expts. no differences existed in body weights between the rats to be used as controls and those to be teeth amputated. The body weights of the teeth amputated rats with decentralized glands decreased ($p < 0.001$) during the 14 days from (mean \pm S.E.) 254 ± 8 to 259 ± 8 g ($n = 9$), i.e. by 12%. During the same time period the control litter mates increased ($p < 0.001$) in weights by 7%, from 293 ± 9 to 314 ± 9 g ($n = 9$). The body weights of the teeth amputated rats with an intact innervation decreased from 301 ± 7 to 267 ± 8 (9) g, i.e. by 11% in the expt. of the duration of 14 days and in the expt.

TABLE I Effects of repeated teeth amputations on the wet weight and choline acetyltransferase activity of submaxillary glands, parasympathetically decentralized or normally innervated. For estimation of the enzyme activity the glands from one rat were pooled. Number of observations is given in brackets. Values are mean \pm S.E.

Days	Experiments	Wet weights mg	Enzyme activity	
			In μ g ACh/h/ pooled glands (total activity)	I μ g ACh/h/ g acedione powder (concentration)
14	Decentralization + Amputations	314.4 \pm 8.7 (18) ⁴	36.7 \pm 1.9 (9)	489 \pm 17 (9) ⁴
	Decentralization	168.9 \pm 4.7 (18)	36.6 \pm 2.4 (9)	547 \pm 23 (9)
14	Amputations	287.4 \pm 8.4 (18) ⁴	51.5 \pm 4.9 (9) ⁴	452 \pm 37 (9) ⁴
	No treatment	200.4 \pm 2.7 (18)	46.3 \pm 3.5 (9)	555 \pm 39 (9)
1	Amputations	358.7 \pm 22.5 (14) ⁴	43.6 \pm 2.7 (7) ⁴	306 \pm 43 (7) ⁴
	No treatment	30.7 \pm 4.7 (14)	37.7 \pm 1.5 (7)	4.6 \pm 18 (7)

¹ > 0.1 < 0.05 < 0.01 ⁴ < 0.001 When the glands of the teeth amputated rat are compared with those of the control litter mate.

of 21 days from 350 ± 10 to 305 ± 9 g ($n=7$), i.e. by 13% the difference is significant at the $p < 0.001$ and < 0.01 levels respectively. In the expt. of 14 days the controls increased ($p < 0.001$) in weights by 12% from 305 ± 6 to 342 ± 6 g ($n=9$), while the weights of the controls in the expt. of 21 days did not change significantly (from 350 ± 9 to 359 ± 6 g, $n=7$).

Gland weights and choline acetyltransferase activity

The effects of teeth amputations on the weights, the total enzyme activity and the enzyme concentration of the submaxillary glands are shown in Table I and in Fig. 1.

Decentralized glands The submaxillaries were 96% heavier (dry weights) in the rats exposed

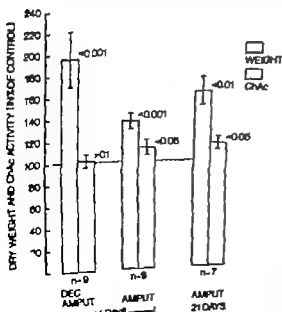


Fig. 1 Dry weight and choline acetyltransferase activity (calculated per pool of 2 glands) of the parasympathetically decentralized or the innervated submaxillary glands after teeth amputations over a period of 14–21 days. The weight and the enzyme activity are expressed as percentage of those of the glands of the control litter mate (mean \pm S.E.). Number of comparisons and the p-values are given.

to teeth amputations than in the rats serving as controls. The total choline acetyltransferase activity did not differ significantly between the glands of the teeth amputated rat and those of the control litter mate. The enzyme concentration was lower in rats after teeth amputations than in controls; this is attributed to the marked gain in weight.

Isolated glands. In the teeth amputated rats the submaxillary glands were 38 and 65% heavier (dry weights) than in the respective controls 14 and 21 days after the beginning of the exp., the difference between the percentage figures was almost significant ($0.05 < p < 0.1$). The total enzyme activity was 13 and 16% higher in the glands of the teeth amputated rats than in the glands of the control rats after 14 and 21 days, respectively; the percentage figures did not differ from each other significantly. The enzyme concentration was lower than in the controls both after the short and the long experimental period.

When the sublingual glands were examined in the rats exposed to teeth amputations over a period of 21 days the dry weight and the total enzyme activity were 135 and 118% of that of the control glands, respectively.

Discussion

In the present study teeth amputations over a period of 2 weeks were found to have left the total choline acetyltransferase activity unchanged in the parasympathetically decentralized submaxillary glands, while the enzyme activity in the glands supplied with an intact innervation is increased by about 13 per cent after the same period of time. It was not further suggested when the amputations continued for another week. It appears from the present study that the choline acetyltransferase in the postganglionic neurones of the submaxillary glands is for its activity dependent on an intact connection with the central nervous system; the enzyme activity increased in glands normally innervated in response to the teeth amputations, while it was not influenced by the treatment when the preganglionic nerve had been sectioned in advance. It appears reasonable to attribute the small increase in enzyme activity of the submaxillary gland to an enhanced stream of impulses in the parasympathetic nerve reflexly evoked by the activation of palpal receptors; this is also the likely explanation to the small increase observed in the sublinguals. In this connection it should be pointed out that the percentage increase in enzyme activity found in some other type of exps., in which the reflex stimulation is thought to have been increased, was also moderate, *i.e.* in the range of 10-30 per cent (see Ekström 1975).

There is no ready explanation to the difference between the result obtained in this study and that in the study of Ohtlin and Peroc (1967), who found in the decentralized glands, enlarged by about the same percentage figure as in the present work and also studied over the same time period, a choline acetyltransferase activity that was twice as high as in the glands of controls. In the two investigations the animals were of the same sex and belonged to the same strain. Of importance for the outcome of the present study may be that comparisons were made between an experimental rat and a control rat of the same age and further more, belonging to the same litter. In a recent report attention was drawn to the fact that the choline acetyltransferase activity varies with the age of the animals (Ekström 1976). It

TABLE I Effects of repeated tooth amputations on the wet weight and choline acetyltransferase activity of submaxillary glands, parasympathetically decentralized or normally innervated. For estimation of the enzyme activity the glands from one rat were pooled. Number of observation is given in brackets. Values are mean \pm S.E.

Days	Experimenta	Wet weights mg	Enzyme activity	
			I μ g ACh/h/ pooled glands (total activity)	II μ g ACh/h/ g acetone powder (concentration)
14	Decentralization + Amputations	312.4 \pm 8.7 (18) ^a	36.7 \pm 1.9 (9) ^a	289 \pm 17 (9) ^a
	Decentralization	168.9 \pm 4.7 (18)	36.6 \pm 4.4 (9)	547 \pm 23 (9)
14	Amputations	287.4 \pm 8.4 (18) ^a	51.5 \pm 2.9 (9) ^a	45 \pm 37 (9) ^a
	No treatment	200.4 \pm 2.7 (18)	46.3 \pm 3.3 (9)	555 \pm 39 (9)
21	Amputations	358.7 \pm 22.5 (14) ^b	43.6 \pm 2.7 (7) ^a	306 \pm 23 (7) ^a
	No treatment	230.7 \pm 4.7 (14)	37.7 \pm 1.5 (7)	426 \pm 18 (7)

^a > 0.1 ^b < 0.05 * < 0.01 < 0.001 When the glands of the teeth amputated rat are compared with those of the control litter mate

of 21 days from 350 \pm 10 to 305 \pm 9 g (n = 7) i.e. by 13% (the difference is significant at the p < 0.001 and < 0.01 levels, respectively). In the expt of 14 days the controls increase (p < 0.001) in weights by 12% from 305 \pm 6 to 342 \pm 6 g (n = 9) while the weights of the controls in the expt of 21 days did not change significantly (from 350 \pm 9 to 359 \pm 6 g, n = 7).

Gland weights and choline acetyltransferase activity

The effects of teeth amputations on the weights, the total enzyme activity and the enzyme concentration of the submaxillary glands are shown in Table I and in Fig. 1.

Decentralized glands: The submaxillaries were 96% heavier (dry weights) in the rats exposed

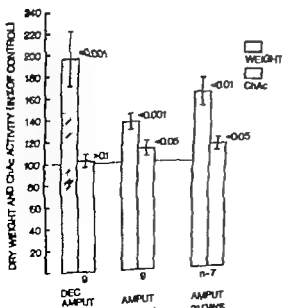


Fig. 1 Dry weight and choline acetyltransferase activity (calculated per pool of 2 glands) of the parasympathetically decentralized or normally innervated submaxillary glands after teeth amputations over a period of 14 or 21 days. The weight and the enzyme activity are expressed as percentage of those of the gland of the control litter mate (mean \pm S.E.). Number of comparisons and the p-values are given.

Carbonic Anhydrase in the Intestinal Tract of the Guinea-pig

By

GUDMAR LÖNNERHOLM

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Abstract

LÖNNERHOLM, G. Carbonic anhydrase in the intestinal tract of the guinea-pig. *Acta physiol. scand.* 1977 99 53-61

The distribution of carbonic anhydrase activity in the intestinal tract of the guinea-pig was studied by the histochemical method of Haneson. Enzyme activity was demonstrated in epithelial cells, erythrocytes and capillary cells. In the gastric mucosa parietal cells, surface mucous cells and neck mucous cells were highly active in the small intestine only a few epithelial cells on the villi and in the upper part of the crypts showed enzyme activity. They seemed to be randomly scattered among inactive cells. It is not clear at present if they represent distinct cell type or specialized absorptive cells. In the proximal colon most surface epithelial cells were highly active (goblet cells are inactive), whereas the surface cells in the distal colon showed less activity with more varying degree of staining. In the caecum enzyme activity was found in the surface epithelium and in the upper part of the crypts, the staining being most marked at the luminal border of the surface cells. The staining reaction was completely inhibited in all tissues by 10 μ M acetazolamide, except for the luminal staining of the caecum, which was inhibited only by 100 μ M acetazolamide. This indicates the presence of high concentrations of carbonic anhydrase, probably of the "low activity" form, at this locus. Mucosal scrapings were taken from the intestinal tissues, homogenized and assayed for carbonic anhydrase activity by changing-pH indicator method. The results confirm those of previous studies and correlate well with the histochemical findings.

Key words: Carbonic anhydrase, histochemistry, stomach, intestine

The presence of carbonic anhydrase in the gastric mucosa is well known, and its distribution and function in this tissue have been extensively studied (see Maren 1967). However much less is known about the enzyme in the other parts of the intestinal tract. Carter and Parsons (1971) found that homogenates of the stomach, proximal colon and caecum of the guinea-pig all possess high carbonic anhydrase activity whereas the activity of the small intestine and distal colon is low. Similar findings have been reported for the dog and rat (Kamaki and Magee 1964; Maren 1967). The physiological significance of these findings is poorly understood, however (see Carter 1972).

The aim of the present study was to clarify the detailed distribution of the enzyme in the intestinal tract of the guinea-pig, since such data are necessary for a further discussion of the role of the enzyme. For this purpose Haneson's (1967, 1968) cobalt-phosphate method for histochemical demonstration of carbonic anhydrase activity has been used. The specificity of this method has been described in another paper (Lönnérholm 1974). Except for

may also have been of importance that the tissue extracts to be compared were incubated simultaneously and that the assay was performed on the same rectus muscle.

The synergistic action of the two branches of the autonomic nervous system in the hypertrophy of the submaxillary glands in rats, the incisors of which were cut was pointed out by Wells and Peronace (1964). The marked gain in weight of the parasympathetically decentralized submaxillary glands shows the role of the sympathetic nerve—the enlargement of the sublingual glands was probably entirely dependent on the parasympathetic nerve, since the secretory cells of this gland are considered to lack an adrenergic nerve supply (Norber and Olson 1965, Ohlin and Perac 1965).

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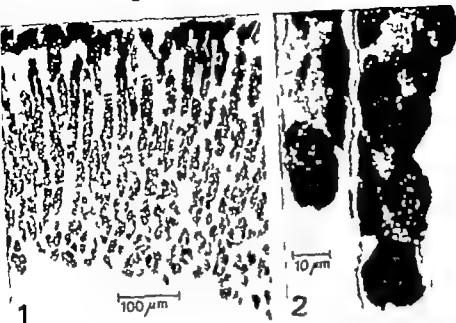


Fig. 1 Gastric mucosa, corpus. Note heavy and uniform staining of the surface epithelium (at the top of the picture). The cells of the glands show varying degree of staining. Fixed, 6 μ m thick section. Incubation time 6 min.

Fig. 2 Gastric glands. Stained parietal cells are seen among unstained or weakly stained chief cells. Fixed, 4 μ m thick section on Millipore filter. Incubation time 9 min.

Stomach. The carbonic anhydrase activity of gastric mucosal homogenates was the highest found in any intestinal tissues (Table I). Histochemically heavy staining was seen in the surface mucous cells, including those lining the gastric pits (Fig. 1). The whole cells were stained, sometimes with an accumulation of the deposits at the cell borders. The neck mucous cells were similarly stained. In the lower part of the gastric glands, the parietal cells

TABLE I Carbonic anhydrase activity of mucosal homogenates.

Tissue	Enzyme units/g wet weight
boile blood ^a	1 280-639
gastric corpus	2 280 \pm 49
pylorus	63 \pm 26
duod.	57-14
cacum	548 \pm 216
proximal colon	1 215 \pm 433
distal colon	96 \pm 21

^a Values are means \pm S.E. for 4 animals.

^b Enzyme units/ml boile blood.

The nomenclature of Ito (1967) has been used.

the stomach, previous histochemical studies are scarce (Korhonen *et al.* 1966, Cassidy and Lightfoot 1974). The enzyme activity of mucosal homogenates of the intestinal tissues was also determined, using a changing pH indicator method.

Methods

Tissue preparation. Nonfasted, female guinea-pigs, weighing 300-600 g, were used. They were fed pellets (Ewos avelsfoder kanin och marsvin, Astra Ewos AB Södertälje, Sweden) and water to which ascorbic acid had been added *ad libitum*. They were killed by a blow on the head. The abdomen and the thorax were rapidly opened and a cannula was introduced into the ascending aorta. Perfusion with cold 0.9% NaCl started after the portal vein had been opened to secure free flow and lasted until the abdominal vessels were macroscopically blood-free.

The stomach and cecum were removed, opened, and the contents washed away by gisting the tissue in a large volume of 0.9% NaCl. Samples were taken from the gastric corpus and from the middle part of the cecum. Intestinal segments were used with or without previous perfusion of the intestinal lumen with 0.9% NaCl. Jejunal samples were taken 5 to 20 cm distal to the duodenojejunal flexure, and ileal samples 5 to 20 cm proximal to the ileocolic valve. Proximal colonic samples were taken 5 to 10 cm distal to the ileocolic valve and distal colonic samples 5 to 10 cm proximal to the anus.

Histochemical staining procedure

Fixed and unfixed tissues from 10 animals were used. Unfixed tissues were immediately frozen in isopentane cooled with a mixture of ethanol and solid CO₂. They were stored in small plastic bags at -70°C for days or weeks before use.

Fixed tissues were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 4-5 hrs. The glutaraldehyde was prepared from 40% stock solution by a one stage vacuum distillation, and the purity of the distillate was checked by ultraviolet spectrophotometry (Anderson 1967). The tissues were then briefly rinsed in 0.2 M sucrose + 0.05 M phosphate buffer (pH 7.4) and frozen as described above.

Sections were cut in a cryostat at -20°C. They were stained for carbonic anhydrase activity according to the cobalt-phosphate method of Hansson (1967, 1968) with only minor modifications. In this method sections are floated on medium containing N HCO_3 , CoSO_4 , KH_2PO_4 and H_2SO_4 . A compound containing cobalt and phosphate is deposited at carbonic anhydrase sites and is converted to CoS . Thus, a black precipitate is formed where enzyme is present. The procedure is described in a paper by Lönnérholm (1974) with discussion of the specificity of the method.

Sections with and without counterstaining with hematoxylin and eosin were prepared.

Throughout the work, the specificity of the staining procedure was checked by incubation of sections in the presence of 10 μM acetazolamide (Diamox® American Cyanamid Company Pearl River NY USA) a specific inhibitor of carbonic anhydrase (Maren 1967).

Biochemical assay of carbonic anhydrase activity

Blood samples were taken by cardiac puncture and collected into a heparinized test tube. The erythrocytes were hemolyzed by adding a large amount of distilled water.

Tissues were prepared as described above, and then gently blotted on absorbent paper to remove excess fluid. Mucosal scrapings were taken with a knife blade. They were weighed and homogenized in 9 parts of distilled water containing 1 mM EDTA (sodium salt) to protect the enzyme from inactivation by heavy metal ions. A tight fitting Teflon plunger in a glass tube was used.

The activity of the hemolysates and the whole homogenates was determined by the change in pH method of Philpot and Philpot (1936) as described in detail by Maren *et al.* (1954). The activity of a sample was calculated from 1-2 determinations on each of 3-4 dilution of the sample. The assays were performed at 2°C.

Results and Discussion

For the histochemical work fixed and unfixed tissues were used as described above. The cellular structure was much better preserved in the fixed sections, allowing more detailed description of the distribution of the enzyme, but the results generally agreed.

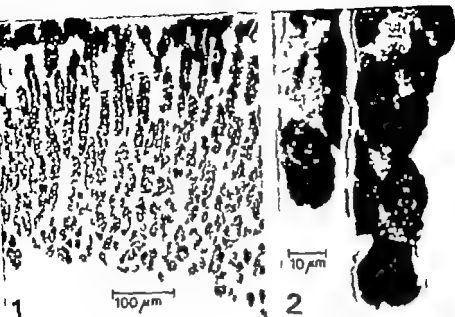


Fig. 1. Gastric mucosa, corpus. Note heavy and uniform staining of the surface epithelium (at the top of the picture). The cells of the glands show varying degree of staining. Fixed, 8 μ m thick section. Incubation time 6 min.

Fig. 2. Gastric glands. Stained parietal cells are seen among unstained or weakly stained chief cells. Fixed, 4 μ m thick section on Mallory filter. Incubation time 9 min.

Stomach. The carbonic anhydrase activity of gastric mucosal homogenates was the highest found in any intestinal tissues (Table I). Histochemically heavy staining was seen in the surface mucous cells, including those lining the gastric pits (Fig. 1). The whole cells were stained, sometimes with an accumulation of the deposits at the cell borders. The neck mucous cells were similarly stained. In the lower part of the gastric glands, the parietal cells

TABLE I. Carbonic anhydrase activity of mucosal homogenates.

Tissue	Enzyme units/g wet weight
boile blood ¹	1 290 \pm 639
gastric corpus	2 280 \pm 492
jejunum	63 \pm 26
duod.	57 \pm 14
cacum	548 \pm 216
proximal colon	1 215 \pm 433
distal colon	96 \pm 21

Values are means \pm S.E. for 4 animals.

¹ Enzyme units/ml whole blood.

The nomenclature of Ito (1967) has been used.

were the most active ones (Fig. 2). Their cytoplasm showed a net-like staining pattern, which probably corresponds to the so-called intracellular canaliculi (see Ito 1967). The chief cells appeared unstained or sometimes weakly stained along the cell borders.

The presence of large amounts of carbonic anhydrase in the mammalian gastric mucosa was an early discovery (Davenport and Fisher 1938). Carter and Parsons (1971) measured the concentration of the two major isoenzymes of carbonic anhydrase in the intestinal tract of the guinea pig, and found that the gastric mucosa contains much "high activity" isoenzyme, but only a negligible amount of "low activity" isoenzyme. The enzyme is supposed to have a role in gastric acid secretion since acetazolamide, a specific inhibitor of the enzyme, reduces the output of hydrogen ions (Janowitz *et al.* 1952).

High enzyme activity in the parietal cells has previously been demonstrated in the guinea pig and other mammals by histochemical (Voilrath 1959; Hansson 1968; Cross 1970; Wärborn *et al.* 1974) and other techniques (Davenport 1939; Boass and Wilson 1964). The present data confirm these findings and give further support for the idea that the parietal cells secrete hydrogen ions.

The present demonstration of considerable carbonic anhydrase activity in the cells of the surface epithelium and the neck mucous cells is in agreement with the findings of Voilrath (1959) in the guinea-pig and Boass and Wilson (1964) in the rat. Others have reported no or low activity in these cells (Davenport 1939; Korhonen *et al.* 1966; Petermann *et al.* 1972). The role for the enzyme in the surface and neck mucous cells is not clear at present. It has recently been shown, however, that the frog fundic mucosa is capable of secreting an alkaline fluid (Flemström 1976). This was demonstrated after the acid secretion had been blocked by inhibitors not affecting carbonic anhydrase. When acetazolamide was added the alkaline secretion was inhibited. These findings, together with the present ones, suggest that carbonic anhydrase in the surface epithelium has a role in alkaline secretion, which may protect these cells from damage due to a low gastric intraluminal pH.

Small intestine. Mucosal homogenates of the jejunum and ileum showed low enzyme activity which was only about 1/40 of that of the stomach (Table 1). The histochemical findings in the jejunum and ileum were similar and are described together.

The epithelium of the villi and the crypts showed no activity except for a small number of cells which were stained distinctly (Fig. 3, 4). These slender and elongated cells were often found to reach from the intestinal lumen to the epithelial basement membrane. They seemed to be fairly evenly distributed on the villi but were less common in the crypts. In the bottom of the crypts no stained cells were found. No attempt was made to quantify the number of stained cells, but a rough estimate is that 1/10 to 1/30 of the cells of the villus epithelium were stained. Obvious differences between the number of stained cells in the different parts of the small intestine were not observed.

The whole cells were stained in fixed sections (Fig. 4) whereas the staining was restricted to the luminal part of the cells in unfixed sections (Fig. 5). This might be due to loss of enzyme from the cytoplasm of unfixed cells.

Carter and Parsons (1971) found small amounts of "high activity" and "low activity" carbonic anhydrase in the small-intestinal mucosa. The present histochemical findings show that the enzyme is localized to a certain population of mucosal cells, scattered in the villus

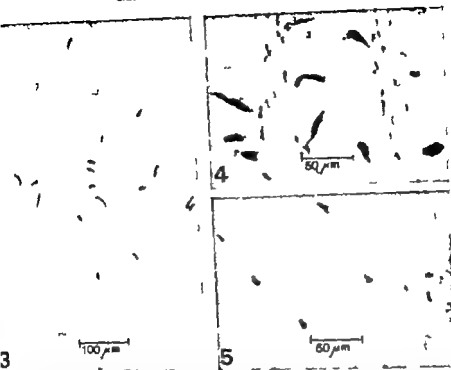


Fig. 3. Mucosa of the distal ileum. A few epithelial cells on the villi and in the upper part of the crypts are stained. Fixed, 8 μ m thick section. Localization time 4 min. Counterstaining with hematoxylin and eosin.

Fig. 4. Lower part of branching villi in the proximal jejunum. A few epithelial cells are stained. When increased longitudinally they can be seen to reach from the epithelial basement membrane to the intestinal lumen. There is also some capillary staining. Fixed, 8 μ m thick section. Localization time 6 min. Counterstaining with hematoxylin and eosin.

Fig. 5. Closely packed villi in distal ileal mucosa. A few epithelial cells are stained at the lateral cell border. Unfixed, 4 μ m thick section on Millipore[®] filter. Localization time 3 min. Counterstaining with hematoxylin and eosin.

epithelium. The low activity of the mucosal homogenates corresponds to the small number of active cells.

Earlier histochemical studies are confined to the small intestine of the rat (Korhonen *et al.* 1966, Cassidy *et al.* 1972, Cassidy and Lightfoot 1974). The results differ from the present ones since these authors reported a uniform staining of the villus epithelium, at least in the jejunum. This discrepancy might possibly be due to the use of different species or techniques.

The identity of the carbonic anhydrase containing cells found here has not been clarified yet. They are obviously not identical with goblet cells, however. They might represent specialized absorptive cells, or a distinct cell type, for example some kind of endocrine cell. Further attempts to characterize the carbonic anhydrase cells by various staining techniques and studies at the ultrastructural level are in progress. The discussion of the role of the enzyme in the small intestinal mucosa must await the result of such studies.

Comment. The carbonic anhydrase activity of the cecal mucosa was about 1/4 of that of the

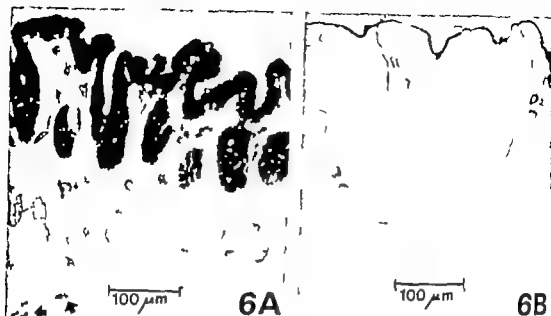


Fig. 6 Cecal mucosa. 6 A The cells of the surface epithelium and the upper part of the crypts are intensely stained. Arrows indicate stained erythrocytes. 6 B Addition of $10 \mu\text{M}$ acetazolamide to the incubation medium inhibits all staining except at the luminal border of the surface cells. Fixed, $8 \mu\text{m}$ thick sections. Incubation time 6 min. Weak counterstaining with hematoxylin and eosin.

gastric mucosa (Table I). Histochemically intense staining was found in the cells of the surface epithelium and the upper part of the crypts, whereas those lining the lower part of the crypts were unstained (Fig. 6 A). With short incubation times the luminal part of the cells showed the most intense staining. With longer times this was obscured by a heavy staining of the whole cells.

The addition of $10 \mu\text{M}$ acetazolamide to the incubation medium blocked the cytoplasmic staining, but not the staining at the luminal cell border of the surface cells (Fig. 6 B). It took $100 \mu\text{M}$ acetazolamide to inhibit this staining. This is remarkable since the low concentration of $10 \mu\text{M}$ of the inhibitor completely abolished the staining in the other intestinal tissues. To exclude the possibility that the luminal staining was non-enzymic and inhibited non-specifically by the high concentration of the inhibitor sections were also incubated without any substrate (*i.e.* bicarbonate) in the medium. This produced completely unstained sections.

Previous histochemical studies of the cecum seem to be lacking. However Carter and Parsons (1971) have reported that the cecal mucosa contains predominantly a "low activity" isoenzyme of carbonic anhydrase, its concentration being higher than in any other intestinal tissue of the guinea pig. This "low activity" isoenzyme is many times less sensitive to acetazolamide than the "high activity" isoenzyme (Carter and Parsons 1972).

That the cecal mucosa indeed possesses considerable enzyme activity is shown here biochemically and histochemically. The present finding that the staining at the luminal border of the surface cells was abolished only by high concentrations of acetazolamide would indicate that high concentrations of carbonic anhydrase, probably of the "low activity" isoenzyme. This localization of the enzyme strongly suggests a role in transport

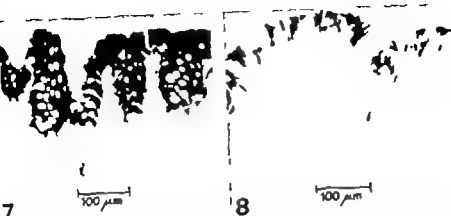


Fig. 7. Proximal colonic mucosa. Intense staining in the surface epithelium and the upper part of the crypts. Unstained goblet cells are seen among the stained cells. Fixed, 8 μ m thick section. Incubation time 6 min. Counterstaining with hematoxylin and eosin.

Fig. 8. Distal colonic mucosa. The surface epithelial cells show varying degree of staining. Fixed, 8 μ m thick section. Incubation time 6 min. Counterstaining with hematoxylin and eosin.

processes between the intestinal lumen and the cells. It has been proposed that the "low activity" carbonic anhydrase of the cecum is concerned with the absorption of products of microbial fermentation, such as ammonia or volatile fatty acids (Carter and Parsons 1971), but this remains hypothetical.

Colon. Carbonic anhydrase activity almost comparable to that of the stomach was found in the proximal colon (Table I). The distal colon showed a much lower enzyme activity. This is in agreement with previous findings in the guinea-pig (Carter and Parsons 1971) and in the dog and rat (Kuraki and Magee 1964).

In the proximal colon heavy staining was found in most cells of the surface epithelium and the upper part of the crypts (Fig. 7). Some unstained, barrel-shaped cells were seen among the stained ones, probably representing goblet cells. The cells in the lower part of the crypts were completely unstained.

In the distal colon the staining of the surface epithelial cells varied markedly. Distinctly stained cells appeared among unstained ones, and intermediate forms showing weak staining are also seen (Fig. 8). The crypt cells were unstained.

These findings agree reasonably well with previous histochemical studies in the rat colon reported by Korhonen *et al.* (1966) and Helander (1975).

The colonic mucosa is capable of absorbing sodium and chloride against large concentration gradients, with a concomitant secretion of bicarbonate. Acetazolamide has been reported to reduce the secretion of bicarbonate and the absorption of sodium, chloride and water in the rat colon (Parsons 1956, Phillips and Schmalz 1970). These findings suggest that carbonic anhydrase may have a role in the exchange of bicarbonate and chloride across the mucosa, and that this anion exchange may be related to the mechanism of sodium and water transport in the colon. The present finding that carbonic anhydrase is localized to the surface cells of the colonic mucosa is in line with such a role for the enzyme.

It is presently not understood, however, why large amounts of the "high activity" and

"low activity" isoenzyme are present in the proximal colonic mucosa, while the distal colonic mucosa possesses only or almost only "low activity" carbonic anhydrase (Ca and Parsons 1971). The role for the "low activity" isoenzyme could possibly be the same as that discussed for the cecal enzyme, i.e. to participate in the absorption of products of microbial fermentation (see above).

Non-epithelial cells Except for epithelial cells, erythrocytes and capillaries were the only stained structures. The few erythrocytes left in the tissues after the perfusion with saline (Methods) were heavily stained (Fig. 6 A). The capillary walls showed a varying degree of staining. This might possibly be due to the presence of a varying number of erythrocytes inside the vessels.

I am grateful to Mrs. Mona Schenholm for skilful technical assistance.

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Regulation of the Heart of a Teleost, *Gadus morhua*, by Autonomic Nerves and Circulating Catecholamines

By

SUSANNE HOLMGREN

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Abstract

HOLMGREN S Regulation of the heart of a teleost *Gadus morhua*, by autonomic nerves circulating catecholamines Acta physiol scand 1977 99 62-74

The cod heart regulation has been investigated by anatomical studies, fluorescent histochemistry stimulation and drug effects on the perfused heart and electrically paced strips. The effect on the catecholamine release from the head kidney has been studied in a perfused head kidney and heart preparation. Branches of the vagi and the fused 1st and 2nd spinal nerves innervate the heart. Special fluorescent fibres were found in these nerve branches, in the ducts of Cuvier all parts of the heart, in arterioles and the ventral aorta. Weakly fluorescent ganglion cells surrounded by specifically fluorescent varicose fibres were present in the walls of the alous venosae. Vagal stimulation caused bradycardia altered after atropine to tachycardia. Spinal nerve and sympathetic chain stimulation also induced tachycardia, as did provoked catecholamine release from the head kidney. The tachycardia could be blocked by propranolol. Atropine increased and propranolol decreased basic heart rate and contraction. Adrenergic drugs accelerated and acetylcholine retarded the perfused heart. A positive inotropic effect was obtained with adrenergic drugs on isolated heart strips, while acetylcholine produced a negative inotropic effect on atrial but not ventricular strips. It is concluded from this study that the cod heart has an inhibitory cholinergic nerve supply via the vagi and an excitatory adrenergic supply via both the vagi and the first spinal nerves. Sympathetically controlled release of catecholamines from the chromaffin tissue in the head kidney may also play an important role in the heart regulation.

Key words: Teleost heart, autonomic innervation, catecholamines

Numerous authors have throughout the years demonstrated the presence of a vagal inhibitory cholinergic innervation of the teleost heart (for references see Von Skramlik 1962, Laurent 1962, Campbell 1970). The cause of excitation of the heart is less clear and seems to vary with species. The possibility of increasing the heart rate by lowering the cholinergic vagal tone has been frequently discussed (McWilliam 1885, Von Skramlik 1935, Kuls 1957, Rodinov 1959, Laurent 1962, Randall 1966). Presence of adrenergic receptors (Brin 1933, Östlund 1954, Falck *et al.* 1966, Gannon and Burnstock 1969, Gannon 1971, Col and Santer 1973) indicates ability of regulation via circulating catecholamines and/or adrenergic nerves. No adrenergic nerves are found in the heart of the plaice, *Pleuronectes platessa* (Santer 1972), and the excitatory effects of vagal stimulation at low frequencies are

proved to be rebound effects (Cobb and Senter 1973). The trout (*Salmo trutta*, *S. trutta*) heart is, however, innervated by sympathetic adrenergic fibres running in the vagus nerve; these are presumed to cause the excitatory effect on vagal stimulation with low frequencies after atropine (Yamauchi and Burnstock 1968, Gannon and Burnstock 1969, Gannon 1971).

In this study the cardiac innervation and the possibility of cardiac regulation by circulating catecholamines in the cod, *Gadus morhua*, have been investigated.

Materials and methods

Cod. *Gadus morhua*, body weight 200–2 000 g, kept in aerated, circulating sea water at 10°C were used in this study. Fish, which were used for perfusion experiments, were before killing injected with heparin 5000 U per kg b.wt. in the caudal vessels. The fish were killed by a sharp blow on the head. A cod Ringer solution (Holmgren and Nilsson 1974) was used as perfusion fluid and saline for isolated strips.

Anatomical studies. For dissection (fish weighing 1–2 kg) are used. They were carefully opened by an incision behind and parallel to the 4th gill arch for studies of the innervation of the heart. 1–2 mg ascorbic acid was used for staining of the nerve fibres (Young 1931).

Fluorescent histochemistry. Tissue pieces were rapidly dissected out from freshly killed fish and quickly put in liquid propane cooled in liquid nitrogen. They were freeze-dried and prepared according to the method described by Palck and Overbo (1945). Since adrenaline is the dominant catecholamine in the heart (Euler and Flügge 1961, Ahrén and Nilsson 1976), 3 h incubation in formaldehyde vapour at 10°C was used. The pieces were embedded in paraffin wax, sections of 10 μ m are cut, mounted in Eriethen (Merck) and viewed in a Leitz-Ortholux microscope with top light illumination and a barrier filter at 460 nm. With this treatment the presence of catecholamines in the preparations could be identified from red-green ('specific') fluorescence. Photographs were taken with a Leitz-Ortholux automatic camera on black Tr-X film.

Perfused head kidney and heart preparations. The fish was opened ventrally: the hepatic veins, the left renal-ovarian vein and the right duct of Coerier ligated and the swim-bladder opened. The inflow catheter (PE 60) was inserted into the left posterior cardinal vein along the length of the swim-bladder up to the head kidney. The perfusion fluid was then allowed to pass through the head kidney and in the duct of Coerier through the heart. An outflow catheter was inserted into the ventral aorta. The catheters were perfused at constant flow rate, achieved with a BRAUN Perfuser with cod Ringer solution. The flow rate used was 0.4 ml/min or 1.0 ml/min depending on animal size. Pressure variations in the outflow of aorta were measured by Statham P 23 transducer. The pulse record was fed into Grass 7P4 Tachograph, which displayed the heart rate as beat-to-beat record. A Grass Polygraph Recorder model 79B was used for the recordings. The sympathetic ganglion, bare fibres from the left sympathetic chain pass through to the chromaffin tissue in the walls of the head kidney parts of the posterior cardinal vein (Nilsson 1976), was carefully located and placed on platinum electrodes for stimulation.

Heart perfusion at constant pressure. The fish was placed ventral side up and opened ventrally and behind the 4th gill arch on each side. The hepatic veins were ligated. Polyethylene catheters (PE 60) were inserted into the left duct of Coerier and the ventral aorta for inflow and outflow respectively of perfusion fluid. Perfusion with cod Ringer solution was performed with constant pressure as described by Nilsson and Gross (1974). The inflow pressure was kept between 5 and 15 cm of water. Within this range the heart rate was found to be independent of the perfusion pressure. Johansen (1962) also reports that the cod heart reacts to increased venous returns with increased beat volume and keeps the heart rate constant. The outflow pressure was kept above 20 cm of water.

When effects of nerve stimulation. were tested the pericard was left intact, and the heart rate was obtained as described above for the perfused head kidney and heart preparation. Platinum electrodes for vagal stimulation were placed either around the whole vagus nerve or 10 mm from the skull or around the entire duct of Coerier or 5 mm from the main venous. Electrodes for stimulation of the branches of the fused 1st and 2nd spinal nerves running parallel to the duct of Coerier were placed between 20 and 30 mm from the outflow of the skull. For stimulation of the right sympathetic chain the right side of the body wall of the fish was cut away. The parts of the sympathetic chain near the coeliac ganglion were dissected free from surrounding head kidney tissue and stimulated with platinum electrodes.

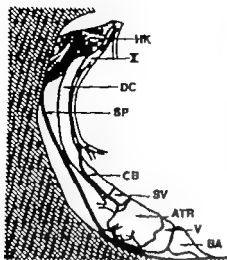


Fig. 1 Lateral view of the right side of the cardiac apex of the cod *Gadus morhua*. The heart is innervated by cardiac branch of the vagus nerve. Fibres have been seen to leave the branch of the fused 1st and 2nd spinal nerves running down along the duct of Cuvier and forer ventral of the pericard, and to enter the duct of Cuvier the atrial tissue directly via the pericard. Legend: ATR=atrium, BA=bulbus arteriosus, CB=cardiac branch of the vagus nerve, DC=duct of Cuvier, SP=branch of the fused 1st and 2nd spinal nerves, SV=sinus venosus, HK=kidney, V=ventricle, X=vagus nerve.

In experiments where drug effects on the heart rate have been tested, the right duct of Cuvier was kept and the pericard opened. A piece of cotton was placed around the ventricle and tied to a Grass FT transducer connected to a Grass Polygraph Recorder model 79B for recording of the heart movement. The heart rate was displayed as a beat-to-beat record by a Grass 7P4 Tachograph.

Agonists were added in single doses to the lower funnel of the perfusion apparatus (Nilsson and Ge 1974) to prevent the drugs from dilution in the major bulk of the perfusion fluid. Antagonists were added to the entire perfusion fluid 15 min before the following nerve stimulation or addition of agonists. Dose-response experiments were made with the agonists and their effects are presented as pD_5 -values (cf. Rossum 1963). Blocking effects of antagonists are, when possible, calculated as pA_2 and pA_{2-3} (Schlid 1947) as described by Holmgren and Nilsson (1975).

Isolated heart strip experiments. Longitudinal strips, ca. 1 mm wide and 5–10 mm long were cut from both atrium and ventricle. They were mounted in organ baths in cod Ringer solution at 10°C, bubbled with a mixture of 3% CO_2 and 97% O_2 , and connected to Grass FT 03 transducers for recording of tension on a Grass Polygraph Recorder model 7 B. Tension was measured in millipond ($mp = 9.81 \times 10^{-4}$). The strips were electrically paced by Grass SD 9 Stimulators with silver electrodes. Cumulative dose-response curves were made, and effects of drugs were calculated as described for the perfused heart.

Drugs. The drugs used were Acetylcholine chloride, 1-adrenaline bitartrate, atropine sulphate, isoprenaline hydrochloride, 1-noradrenaline bitartrate, propranolol hydrochloride.

Statistics. Means are given \pm standard error (S.E.), numbers of expts. are indicated as *n*. Not more than one experiment is done on one fish, except for the isolated strips, where two strips were taken from each heart chamber. Significance of differences in potency between drugs has been calculated by Student *t*-test.

Results

Dissection with osmic acid

The cod heart is innervated by a cardiac branch of each vagus nerve. The fibres follow the duct of Cuvier on both sides into the pericard and are spread out on the sinus venosus, one part on the dorsal and one part on the ventral side, in a region near the atrium (Fig. 1).

Fluorescence histochemistry

Bundles of specifically fluorescent fibres are found both in the branches of the fused 1st and 2nd spinal nerves running along the ducts of Cuvier (Fig. 2 a) and in the cardiac branches of the vagi (Fig. 2 b).

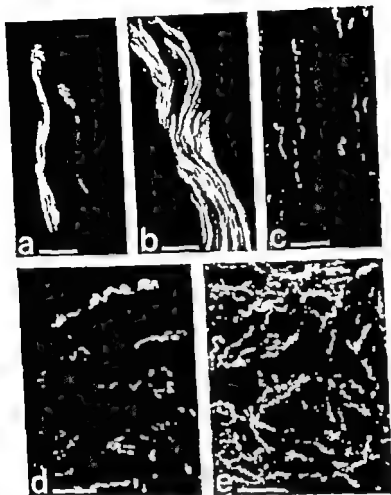


Fig. 3. Microphotographs of fluorescent histochemical preparations. Formaldehyde treatment for 3 h. Longitudinal section of the branch of the fused 1st and 2nd spinal nerves running parallel to the duct of Cuvier shows bundles of specifically fluorescent fibers. b. Longitudinal section of the cardiac branch of the vagus nerve shows bundles of specifically fluorescent fibers running amongst non-fluorescent fibers. d and e. Smooth and varicose fluorescent fibers are bundled in the walls of the ducts of Cuvier (d), the main venous (d) and the bulbus arteriosus (e). Calibration bar in all pictures 90 μ m.

The walls of the ducts of Cuvier as well as the walls of sinus venosus are densely innervated by specifically fluorescent, varicose fibres (Fig. 2 c, 2 d). In the part of sinus venosus bordering to the atrium, aggregations of ganglion cells are located. These seem to lack specific fluorescence themselves, but are surrounded by fluorescent nerve terminals (Fig. 3 a).

The tissues of the atrium and the ventricle show a heavy autofluorescence, which made it difficult to find and photograph the weaker specific fluorescence from the catecholamines. However fluorescent fibres are found throughout the atrial tissue, being most frequently found in the outermost layer of the atrium and in the parts nearest sinus venosus (Fig. 3 b). Fluorescent fibres are also seen running between the atrial and the ventricular tissue (Fig.

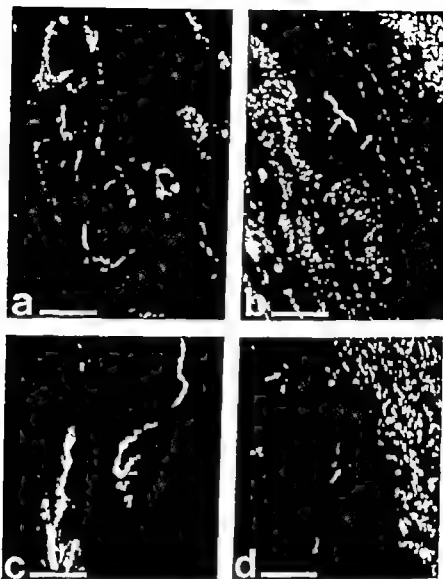


Fig. 3. Microphotographs of fluorescent histochemical preparations of the cod heart. Formaldehyde fixation for 3 h. *a*. Aggregation of ganglion cells (the wall of sinus venosus, bordering the atrial tissue, right corner). The ganglion cells lack specific fluorescence themselves, but are surrounded by specific fluorescent varicose fibres. *b*. The atrial tissue shows heavy autofluorescence, but specifically fluorescent fibres can be seen (arrows). *c*. Specific fluorescence in the tissues surrounding the opening between atrium and the ventricle. *d*. Varicose specifically fluorescent fibres (arrows) in the outermost part of ventricle, where the autofluorescence is absent.

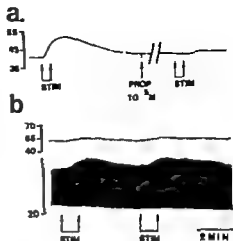
3 c) The ventricular tissue is much less innervated by fluorescent fibres than the atrial tissue, but specifically fluorescent terminals are found especially in the outermost part (Fig. 3 d).

Bulbus arteriosus (Fig. 2 e) and the ventral aorta are densely innervated by specifically fluorescent fibres.

Perfused head kidney and heart preparation

The heart rate during perfusion with constant flow rate through the head kidney and heart was in 15 fish tested 49 ± 3 bpm. In 11 of these fish stimulation of the satellite ganglion

Fig. 4. Effects on two different cod hearts of stimulation of the nerves to the chronotropic tissue in the nodal body during perfusion of the head kidney and the heart. Time bar = 2 min. a. Recording of the heart rate. Stimulation (STIM) with 20 Hz, 1 ms and 10 V for 30 s, before and 15 min after addition of propranolol to final concentration of 10^{-6} M the perfusion fluid. b. Upper tracing: heart rate, lower tracing: outflow pressure. The diastolic outflow pressure was adjusted to 20 cm H₂O or above. No measuring of the absolute 'systolic' outflow pressure was made due to the inaccuracy caused by the experimental setup and since only relative variations in contraction force were of interest for this study. Stimulation with 20 Hz, 1 ms and 20 V for 60 s. A more pronounced autotropic effect is noticed when the chronotropic effect is low.



20 Hz, 1 ms and 10 V for 30 s caused tachycardia (Fig. 4 a), and sometimes an increase in outflow pressure from the heart (Fig. 4 b). The increase in heart rate after the first stimulation varied from fish to fish between -4 bpm and $+25$ bpm (mean value $+11 \pm 2$ bpm). The stimulation effect could be repeated several times, but with a slow decline in the increase in heart rate. Slight responses could in the other 4 fishes be obtained with increased outflow pressure and stimulation time. The increase in outflow pressure (Fig. 4 b) was more pronounced when the effect on the heart rate was low.

Propranolol 10^{-6} – 10^{-8} M abolished the effects on the heart rate after stimulation of the sympathetic ganglion ($n = 4$, Fig. 4 a). Propranolol 10^{-6} – 10^{-8} M decreased the spontaneous heart rate 9 ± 2 bpm ($n = 4$).

vagal stimulation

Stimulation of the vagal branches to the heart with 1 ms and 4–10 V with stimulation frequencies from 0.5 Hz and upwards caused bradycardia (Fig. 5 a). A stimulation frequency 12 ± 3 Hz ($n = 5$) (left vagus) or 8 ± 1 Hz ($n = 6$) (right vagus) arrested the heart beat completely for a stimulation period of 30 s. Tachycardia was never obtained at any frequency tested on the untreated heart. On cessation of vagal inhibition of the heart a transient increase in heart rate compared to before the stimulation was often observed (Fig. 5 b).

After atropine (10^{-6} M) vagal stimulation induced a positive chronotropic and/or inotropic effect on the heart in 7 out of 15 fish (Fig. 5 c, 5 d). Propranolol (10^{-6} – 10^{-8} M) blocked these effects ($n = 4$).

Stimulation of the spinal nerve with 20 Hz, 1 ms and 10–20 V accelerated the heart in 10 out of 18 fish (Fig. 6 a). Stimulation with low frequencies gave less effect than 10 or 20 Hz. The increase in heart rate after stimulation was abolished by addition of propranolol (10^{-6} M, $n = 4$) to the perfusion fluid (Fig. 6 b).

When the spinal nerve and the atropinized vagus were tested in the same animal, either one of them, both or none gave a positive chronotropic effect.

Stimulation of the sympathetic chain behind the coeliac ganglion with varying frequencies and duration never affected the heart ($n = 9$). In 3 fish of 12 tested stimulation at the level

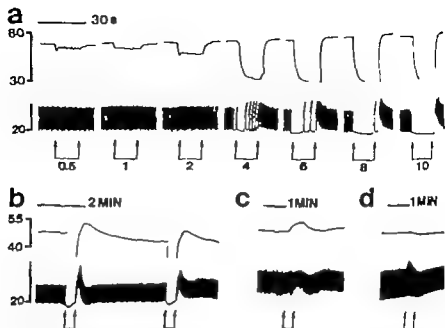


Fig. 5 Effects on the perfused cod heart of stimulation of the vagus nerve. Upper tracings, heart rate. Lower tracings, recording of outflow pressure. The diastolic outflow pressure was adjusted to 30 cm H₂O or above. No measuring of the absolute systolic outflow pressure was made due to the inaccuracy caused by the experimental setup and since only the relative variations in contraction force were of interest for this study. Arrows indicate stimulation period. *a*, Stimulation of the cardiac branch 5 mm from the sinus venosus with 1 ms and 10 V for 15 s. Numbers indicate the stimulation frequency (Hz) used. Heart rate is obtained at all frequencies tested. *b*, Stimulation of the left vagus with 20 Hz, 1 ms and 20 V for 30 s. On cessation of vagal stimulation, a transient increase in heart rate above normal is achieved (Can also be noticed in *a*). *c*, Stimulation of the left vagus with 10 Hz (*c*) or 20 Hz (*d*), 1 ms and 20 V for 30 s. 15 min after addition of atropine to a final concentration of 10^{-6} M in the perfusion fluid, a chronotropic effect was obtained (*c*) while in *d*, an inotropic effect can be noticed.

of the coeliac ganglion with 20 Hz, 1 ms and 20 V caused a slight increase in the heart rate (Fig. 6 c)

Chronotropic effects of drugs

Addition of acetylcholine in single doses to the perfusion fluid caused a dose-dependent decrease in heart rate (Fig. 7 a) with $pD = 8.27 \pm 0.20$ ($n = 13$). Atropine blocked this effect ($pA = 9.8 \pm 0.2$, $pA = 8.9 \pm 0.2$, $n = 6$). Atropine 10^{-6} M also increased the basic heart rate (Fig. 8 b) with up to 15 bpm (mean 5 ± 1 bpm, $n = 16$).

Adrenergic drugs dose-dependently increased the heart rate (Fig. 8 c) with the order of potency isoprenaline ($pD = 10.33 \pm 0.36$, $n = 5$) > adrenaline ($pD = 10.16 \pm 0.14$, $n = 6$) > noradrenaline ($pD = 9.22 \pm 0.19$, $n = 7$, $p < 0.005$ compared to adrenaline) indicating an action via β -adrenergic receptors. The effects of high doses of adrenergic drugs could not be completely washed out, and subsequent dose-response curves showed an irregular decrease in sensitivity. No experiments with adrenergic blocking agents have therefore been performed.

Inotropic effects of drugs

Inotropic effects of drugs on the heart were tested on isolated strips, which were paced electrically. Stimulation with 1 Hz, 1 ms and 10–20 V for the atria and 0.5 Hz, 1 ms and 10–20 V for the ventricles was found to pace the strips evenly throughout an experiment.

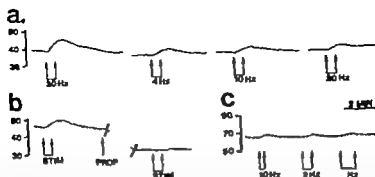


Fig. 6 Recordings of the heart rate of the perfused cod heart. Time bar = 1 sec. a. Stimulation of the spinal nerve every 5th minute with varying frequency (Hz), 1 sec duration and 10 V for 30 sec caused tachycardia. Note the decrease in effect of subsequent stimulations by comparing the 1st and the 4th stimulation. b. The effects of stimulation of the spinal nerve with 20 Hz, 1 sec and 10 V for 30 sec before and 15 min after addition of propranolol to the perfusion fluid. c. Addition of 10^{-6} M atropine to the perfusion fluid. The addition of propranolol in this case also produced pronounced decrease in basic heart rate. Stimulation of the sympathetic chain at the level of the coeliac ganglion with 1 sec and 20 V for 30 sec with 2 Hz and 90 sec with 4 Hz, caused slight increase in heart rate.

1. Addition of atropine to the bath increased the contraction force of the atrial strips with totally $12 \pm 2\%$ ($n = 8$) and $20 \pm 5\%$ ($n = 6$) after a final atropine concentration in the bath of 10^{-6} M and 10^{-5} M respectively (Fig. 8). The ventricular strips were unaffected by atropine up to 10^{-5} M ($n = 8$).

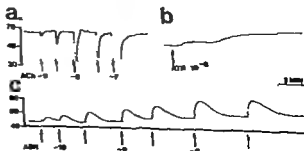
2. Propranolol 10^{-6} M in the bath decreased the contraction force of the atrial strips with $9 \pm 3\%$ ($n = 11$) and of the ventricular strips with $4 \pm 1\%$ ($n = 11$).

3. Cumulative doses of acetylcholine produced a dose-dependent negative inotropic effect on strips from the atria ($pD = 5.19 \pm 0.17$ ($n = 10$), $7 \pm 2\%$ ($n = 10$) of the original contraction caused by the electrical stimulation remained after maximal effect of acetylcholine (Fig. 8).

4. Atropine produced a dose-dependent parallel shift to the right of the acetylcholine curve with $pA_{50} = 9.0$ and $pA_{50} = 8.1$ ($n = 7$). Acetylcholine 10^{-12} – 10^{-6} M had no recordable effects on strips from the ventricle ($n = 11$) (Fig. 8).

5. Adrenergic drugs produced an increase of the contraction force in both atrial and ventricular strips (Fig. 8), with the order of potency isoprenaline > adrenaline > noreadrenaline for

Fig. 7 Chronotropic effects of drugs on the isolated cod heart, perfused *in situ*. Legend: ACh, acetylcholine, ADR, adrenaline, ATR, atropine. Arrows indicate addition of drugs. Agonists are added in subsequent doses of 10^{-11} moles, 10^{-10} moles, 10^{-9} moles, 10^{-8} moles, etc. Time bar = 2 min.



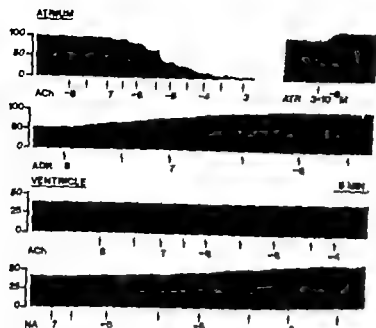


Fig. 8. Inotropic effects of drug on isolated strips from atria or ventricles, electrically paced at 1 Hz, 1 ms and 10 V and 0.1 Hz, 1 ms and 10 V respectively. Legend: ACh = acetylcholine, ADR = adrenaline, NA = noradrenaline, ATR = atropine. Arrows indicate addition of drugs. Arrows indicate addition of drugs. Arrows indicate addition of drugs, giving final concentration to the bath of 10^{-6} M, 3×10^{-6} M, 10^{-7} M, 3×10^{-7} M etc. The tension is given in mP. Time per 5 min.

atrial strips and isoprenaline > adrenaline > noradrenaline for ventricular strips, indicating an effect via β -adrenoceptors. pD_2 -values are presented in Table 1. The effects on the ventricle were, however, irregular from fish to fish: about 50% of the fish tested gave no recordable response at all.

The effect from the first dose-response curve could seldom be completely washed out, and subsequent dose response curves showed a decrease in positive inotropic effects. Attempts to quantify the effects of blocking agents have therefore not succeeded.

Discussion

The results from this study indicate the presence of a vagal inhibitory cholinergic nerve supply to the cod heart, acting on muscarinic receptors in the heart tissue. This is consistent with the situation in other teleosts tested, e.g. the trout, *Salmo trutta* (Gannon and Burnstock 1969, Gannon 1971) and the plaice, *Pleuronectes platessa* (Cobb and Samer 1973) (for references see also Laurent 1962, Burnstock 1969). Both chronotropic effects (whole perfused heart) and inotropic effects (only on atrial strips) are obtained with acetylcholine.

TABLE 1. pD_2 -values for the positive inotropic effect on electrically paced atrial and ventricular cod heart strips, produced by cumulative doses of adrenergic drugs. Values are given \pm S.E., numbers within parentheses indicate number of experiments. Significant difference in potency compared to adrenaline is calculated: $p < 0.05$, $p < 0.01$, $p < 0.001$.

	Isoprenaline	Adrenaline	Noradrenaline
Atrial strips	8.19 ± 0.11 (8)	6.63 ± 0.19 (10)	5.84 ± 0.21 (9)
Ventricular strips	8.49 ± 0.39 (3)	6.94 ± 0.14 (6)	6.53 ± 0.6 (9)

the cod. Ventricular strips were, however, insensitive to acetylcholine, indicating the absence of cholinergic receptors for regulation of contraction force in the ventricle. This sensitivity to acetylcholine, as well as an absence of a cholinergic-inhibitory innervation, also reported for the trout (*Salmo trutta*) and the eel (*Anguilla anguilla*) ventricles (Gannon 1971).

Contrary to the trout and plaice hearts (Gannon and Burnstock 1969, Cobb and Santer 1973), the cod heart reacted to stimulation of the vagi with a decrease in heart rate even at very low stimulation frequencies. A positive chronotropic effect of vagus stimulation was only obtained after atropinization. This effect, which is blocked by the β -adrenergic blocking agent propranolol, is probably due to release of catecholamines from adrenergic varicosities in the vagi. The fluorescent histochemistry gives evidence for the presence of such varicosities in the vagal cardiac branches. Adrenergic terminals are also found in the walls of the sinus venosus, the atrium and the ventricle, showing the existence of an adrenergic innervation. A functioning adrenergic excitatory innervation of the heart has previously been described in trout, *Salmo trutta* and eel, *Anguilla anguilla* (Gannon and Burnstock 1969, Gannon 1971).

The transient cardioacceleration often obtained after cessation of inhibitory vagal stimulation or after inhibition of the heart with acetylcholine might be due to a rebound phenomenon, similar to that described in the plaice heart by Cobb and Santer (1973). Pooling of perfusion fluid in the sinus venosus and duct of Cuvier during the inhibition of the heart activity might also influence the heart rate on cessation of this inhibition (Laurent 1962), although Johansen (1962) reports that variations in venous return does not affect the cod heart rate.

Cardiac acceleration could also be induced by stimulation of the fused 1st and 2nd spinal nerves, the blockade of this effect with propranolol indicates an action via adrenergic nerves. Fluorescent histochemistry also revealed the presence of such fibres in the branch of the first spinal nerves stimulated. The adrenergic fibres may reach the heart via the very fine nerves seen to leave the spinal nerve trunk and to enter the duct of Cuvier or the pericard in some of the dissected fish. Another possible pathway for the nerve fibres is via the ventral aorta and the bulbus arteriosus, which are heavily innervated by adrenergic nerves, and further into the cardiac tissue (Gannon and Burnstock 1969).

The adrenergic innervation to the heart thus seems to run partly in the vagi and partly in the first spinal nerves, the proportions being different from fish to fish. The lack of effect of stimulation of either one or both of the two types of nerves which was often obtained, can, however, not be directly interpreted as a total lack of adrenergic innervation via that nerve. Damages of tissues during the preparation might interfere with the results. Neither were any of the nerves treated for fluorescence histochemistry completely devoid of adrenergic fibres, although it could not be determined whether these fibres reached the heart or not.

The adrenergic fibres reaching the heart via the vagi or the first spinal nerves are most likely of sympathetic origin, leaving the sympathetic chain at the ganglia in connection with the respective nerves (Nilsson 1976). Stimulation of the sympathetic chain also in some cases produced slight acceleration of the heart.

The effect of the adrenergic nerves is, at least partly, direct on the β -adrenoceptors of the

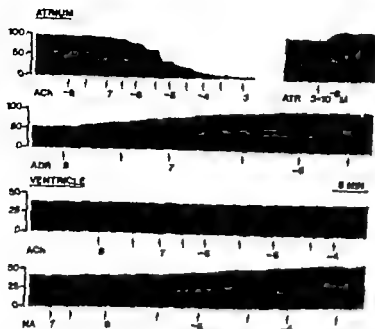


Fig. 2. Inotropic effects of drug on isolated strips from atrium and ventricles, electrically paced at 1 Hz, 1 ms and 10 V and 0.5 Hz, 1 ms and 10 V respectively. Legend: ACh—acetylcholine, ADR—adrenaline, NA—noradrenaline, ATR—atropine. Arrows indicate addition of drugs. Arrows also indicate cumulative doses, giving final concentration in the bath of 10^{-4} M, 3×10^{-4} M, 10^{-7} M, 3×10^{-7} M etc. Tension is given in mm. Time bar 5 min.

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The conclusions from this study are that the cod heart receives a double antagonistic autonomic innervation. Like in other teleosts cholinergic inhibitory fibres, acting on muscarinic receptors, reach the heart via the cardiac branches of the vagi. The presence of an excitatory adrenergic innervation acting on β -adrenoceptors in the cod heart tissue is suggested from histochemical and pharmacological data. These fibres, which are most likely of sympathetic origin, run both in the vagi and in the fused 1st and 2nd spinal nerves, and appear to reach the sinus venosus and both atrial and ventricular tissue. Adrenergic axons may also play a regulating role in the sinus venosus ganglion. Apart from the vagus, catecholamines released from the chromaffin tissue in the head kidney may have an important regulating capacity.

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heart muscle fibres. Specifically fluorescent varicose fibres are frequent in the atria, less common but existent in the ventricle. In both chambers the innervation seemed to be more dense in the outermost parts. This, however could be due to "shading" by the more intense autofluorescence in the rest of the tissue.

Part of the effect of the adrenergic nerves might be a regulation of the non-adrenergic (cholinergic?) ganglion cells in the sinus venosus. These are in fluorescence preparations seen to be surrounded by a network of adrenergic terminals. This has earlier been reported for mammals (Ehinger *et al.* 1968 Nielsen and Owman 1968).

The presence of a tonic activity in the nerves to the fish heart has been much discussed (McWilliam 1885 Von Skramlik 1935 Kulaev 1957 Rodinov 1959 Laurent 1962, Raul 1966). In this study atropine increases and propranolol decreases the heart rate of the perfused heart indicating a both cholinergic and adrenergic tonus in the preparations used. Whether this is due to a normal tonic activity of the nerves or to release and leakage from damaged nerve fibres has not been further investigated. The possibility of direct effects of the blocking agents can neither be completely dismissed, although low doses, especially of atropine, have been used. The effects of atropine and propranolol on the isolated strip might be due either to direct effects of the drugs or to blockade of the effect of transmitter leaking from the cut nerve endings.

Stimulation of the nerves to the chromaffin tissue in the head kidney releases catecholamines, mainly adrenaline (Nilsson *et al.* 1976) into the posterior cardinal vein. In the present experiments, where the outflow of perfusion fluid from the head kidney was allowed to pass through the heart, stimulation of the same nerves caused tachycardia. The effect could be blocked by propranolol and may therefore be regarded due to the above mentioned release of catecholamines. The effect on the heart was much bigger than the acceleration of the heart caused by direct nerve stimulation. It can therefore be considered that release of catecholamines from the chromaffin tissue in the head kidney may play an important role in the regulation of the cod heart. This is contrary to the situation in mammals, where locally released neurotransmitters are found to be dominating in the control of the heart (see Rushmer 1962). It can also be noticed that the concentration range for the complete dose-response curve for adrenaline on the isolated cod atrial strips is 10^{-8} – 10^{-6} M 0.18–1.1 $\mu\text{g}/100$ ml saline. This agrees well with the values of adrenaline content in blood samples from controls and stressed cod, 1 $\mu\text{g}/100$ ml and 4 $\mu\text{g}/100$ ml respectively obtained by Nilsson *et al.* (1976) especially since it is most possible that the concentration of catecholamines reaching the heart directly from the head kidney *in vivo* is higher than the concentration in blood samples taken from the caudal vessels.

The effect of added adrenergic drugs has been difficult to abolish completely by washing out. The catecholamines may somehow be retained in the heart tissue maybe in the catecholamine containing endothelial cells described in the cod atria (Sætersdal *et al.* 1974) and from there slowly released.

In the perfused heart a positive inotropic effect of nerve stimulation or catecholamine release usually can be noticed only when the positive chronotropic effect is weak. When the chronotropic effect is pronounced this may in the preparation used, due to limitations in the method mask an eventual inotropic effect.

Potentiation by Carbachol and Aminophylline of Histamine- and db-cAMP Induced Parietal Cell Activity in Isolated Gastric Glands

By

THOMAS BERGLINDH

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Abstract

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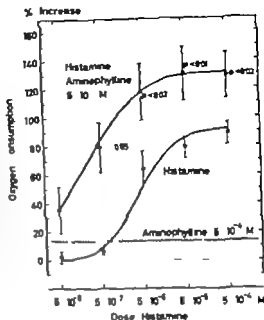
The response to combinations of gastric acid secretagogues was studied in isolated glands from the rabbit gastric mucosa in terms of changes in oxygen consumption and accumulation of the weak base antacopyrine (AP). The latter reflects the acid secreting state of the glands. The following secretagogues were investigated: histamine, carbachol, aminophylline and db-cAMP. The histamine respiratory dose-response curve was shifted to the left in the presence of the phosphodiesterase inhibitor aminophylline. Both ED-50 and maximum response were significantly increased. Histamine-induced AP accumulation was also strongly enhanced by aminophylline ($5 \cdot 10^{-4}$ M). These results are consistent with the hypothesis that histamine stimulation of acid secretion is mediated by cyclic AMP. Carbachol-enhanced oxygen consumption could not be potentiated by aminophylline and the combined effect was only additive. The response to combinations of histamine and carbachol was a significant increase in oxygen consumption above what could be expected from an additive effect alone. Carbachol addition to glands pretreated with histamine gave rapid increases in the respiratory rate resulting in a new steady state level within 10-15 min, as compared with time constant of about 40 min when both drugs were added simultaneously. Likewise AP accumulation increased more rapidly and reached higher values after addition of histamine + carbachol as compared with histamine alone. The db-cAMP-enhanced oxygen consumption was in all respects equally affected by carbachol as was histamine stimulation. This indicates that the well known cholinergic potentiation of histamine stimulation is not due to an increased sensitivity of the histamine receptor but is of more general nature. A mechanism involving intracellular availability of Ca^{2+} is proposed as one possible explanation of this potentiation.

Key words: Gastric glands, histamine, carbachol, db-cAMP, aminophylline, oxygen consumption, antacopyrine.

Through several investigations, the preparation of isolated glands from the rabbit gastric mucosa has proved to be a useful preparation for the study of acid secretory mechanisms in the mammalian parietal cell (Berglindh and Öbrink 1976, Berglindh *et al.* 1976, Berglindh 1976).

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Fig. 1. Dose-response relationship between histamine concentration and oxygen consumption, with or without aminophylline (5×10^{-4} M). The vertical bars represent \pm S.E. (n = 5). The basal gland oxygen consumption was $14 \mu\text{mol/g dry wt. in 60 min}$. Aminophylline per se increased the respiration rate. The p-values indicate whether the response to concentration stimulation with histamine and aminophylline was significantly greater than the algebraic sum of the effects of the two drugs ($p < 0.05$ is considered not significant). The curves are drawn according to the integral of the normal reliability curve obtained by probit analysis.



time response curve was shifted to the left, changing the ED_{50} from 1.1×10^{-6} M to 2×10^{-7} M. Aminophylline alone increased the rate of respiration 13.3%. The histamine + aminophylline-induced increase in respiration was, however, significantly higher than the sum of histamine and aminophylline alone at histamine concentrations from 5×10^{-6} M and higher. Since the maximum response was also significantly enhanced, the effects could be defined as a potentiation according to Cooke (1969) and Hirschowitz *et al.* (1973).

Carbachol and aminophylline Carbachol (6.9×10^{-4} M) and aminophylline (5×10^{-4} M) alone or in combination were added to the gland suspensions and the oxygen consumption studied for 90 min. The percentage increase in oxygen consumption was ($n = 5$ mean \pm S.E.): carbachol 24.7 ± 6.4 , aminophylline 9.0 ± 7.0 , carbachol + aminophylline 36.1 ± 11.5 %. Thus, in this type of study the combined response did not exceed a direct additive value.

Since, however, carbachol stimulation of isolated glands is of a transient nature with a peak value after approximately 15 min followed by a rapid decline (Berglindh *et al.* 1976), aminophylline was also investigated in that situation. The glands were preincubated with aminophylline 5×10^{-4} M and after a 15 min recording period, carbachol was added from the side-arm. As seen from the time-response curves in Fig. 2, basal respiration was stimulated by aminophylline, but the drug did not change the transient response after carbachol per se. Thus the phosphodiesterase inhibitor does not seem to potentiate carbachol stimulation or alter the transient course.

Histamine and carbachol. Histamine and carbachol alone or in combination were added to glands at the start of the incubation. The results are presented in Table I.

With a background of 4.5×10^{-4} M carbachol, histamine in increasing concentrations (up to 1.1×10^{-4} M) always significantly increased respiration above that which could be

Histamine was shown to be a potent secretagogue for the parietal cell in the acid glands, and its actions were mimicked by db-cAMP (Berglindh *et al* 1976). In the present investigation the histamine-cyclic AMP relationship was further studied by the use of phosphodiesterase inhibitor aminophylline.

In previous investigations on the gastric mucosa, histamine has been shown to stimulate adenylate cyclase activity in all cases whereas simultaneously studied cholinergic drugs have produced divergent results, perhaps due to species differences (Nakashima *et al* 1970, Perrier and Laster 1970, Ray and Forte 1974, Sung *et al* 1973). Therefore the glandular responses following carbachol were also investigated in the presence of aminophylline.

In the intact stomach there is a pronounced potentiating effect between histamine and cholinergic stimuli and also normally a cholinergic stimulatory tonus as a background (Andersson and Grossman 1965, Cooke 1969, Hirschowitz and Hutchison 1975). In contrast to *in vitro* conditions, where the effect on H⁺-secretion is sustained, carbachol induces a transient response in the glandular preparation (Berglindh *et al* 1976). Thus in an attempt to approach *in vivo* conditions, the combined effect of histamine and carbachol was studied. Finally also the influence of carbachol upon db-cAMP-stimulated glands was investigated.

Materials and methods

Gastric glands. The glands were prepared from rabbit gastric mucosa according to the method of Berglindh and Öbrink (1976).

Chemicals. Carbachol (carbamylcholine chloride), N⁶,O²-dibutyryl adenosine 3',5'-cyclic phosphoric acid (db-cAMP grade II) as sodium salt and Aminophylline were obtained from Sigma. Histamine dihydrochloride was a product of Schuchardt (Munich).

Aminophylline (N-4-dimethyl-C14) with a specific activity of 3.9 mCi/mmol was purchased from NEN Chemicals.

Respiratory medium. NaCl 132.4 mM, KCl 5.4 mM, Na₂HPO₄ 5.0 mM, NaH₂PO₄ 1.0 mM, MgSO₄ 1.0 mM, CaCl₂ 1.0 mM, phenol red 10 mg/l, pH 7.4. Before use 2 mg/ml rabbit albumin (Sigma) and 100 mg/ml glucose were added.

Respiratory measurements. The oxygen consumption was determined in a Warburg respirometer employing the same techniques as described by Berglindh and Öbrink (1976) and Berglindh *et al* (1976). Either the drugs were added at the start of the incubation or from the side-arm of the Warburg flasks to preincubated glands. All drugs were dissolved in the respiratory medium and added to obtain the desired concentration in 3.0 ml glandular suspension.

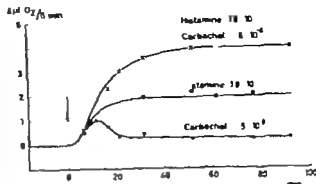
Aminophylline (AP) accumulation. The theoretical and practical considerations concerning the use of the aminophylline for indirect measurements of acid production have been presented by Berglindh *et al* (1976) and Berglindh (1976). In the present investigation AP accumulation kinetics were studied by determinations of the AP concentration in the extraglandular water which very clearly reflects the glandular uptake of AP as well as the accumulation ratio (AP in intraglandular water/AP in extraglandular water). Both methods were described by Berglindh (1976).

Results

Respiratory studies

Histamine and aminophylline. Histamine was added in increasing concentrations to glands taken from the same preparation, with or without a simultaneous addition of aminophylline. 5–10⁻⁴ M. After 30 min preincubation the oxygen consumption was studied for 60 min. The dose-response curves are presented in Fig. 1. In the presence of

Fig. 3. The basic responses of granular oxygen consumption to addition of carbachol, histamine and carbachol-histamine. The drugs were added to preincubated glands as indicated by the arrow. The curves are drawn by eye. A representative example from one of 5 experiments is shown.



considerably higher than could be expected from any type of additive effect. In all experiments, however, the combined response seemed to reach steady state later than did the histamine response.

Quite another type of response occurred when carbachol was added to glands prestimulated with histamine. The steady state histamine response increased almost instantaneously and reached a new steady state level within 10-15 min. An example of such a response is presented in Fig. 4, which shows the difference between the histamine-induced respiration and the carbachol modulated one.

db-cAMP and carbachol. Two types of oxygen consumption studies were performed.

a) **db-cAMP (5×10^{-4} M)** was added to glands prestimulated with carbachol (4.5×10^{-4} M) and the response was compared to that of db-cAMP alone. As seen in Fig. 5 the presence of carbachol enhanced the db-cAMP-response throughout the stimulatory course and gave a steady state value, which was higher than the additive effect of db-cAMP and carbachol. The combined effect of db-cAMP and carbachol appeared, however, to have a longer time constant than that of db-cAMP alone, before reaching steady state value. A simultaneous addition of carbachol and db-cAMP (compare method Fig. 3) gave a response, which was indistinguishable from that described above.

b) When, on the other hand, glands were prestimulated with db-cAMP (5×10^{-4} M)

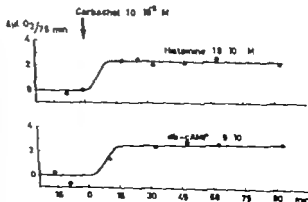


Fig. 4. Carbachol induced respiratory increase in glands prestimulated with histamine or db-cAMP. The figure shows the difference between the carbachol stimulated preparations and their respective controls, stimulated by histamine and db-cAMP. The results are obtained simultaneously from the same gland preparation. The curves are drawn by eye. A typical example from one of 3 experiments is shown.

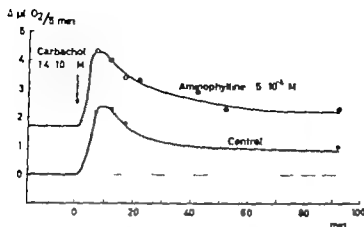


Fig. 2. The transient respiratory response after carbachol in the effect of aminophylline upon stimulatory course. Carb. was added as indicated in treated glands (control) or glands preincubated with aminophylline. The curves are typical examples of 4 expts. in above.

expected from an additive effect alone. Linear transformation of the results gave for his a calculated $V_{\max} = 139.4 \pm 9.2\%$ and $ED_{50} = 7.06 \pm 1.61 \cdot 10^{-4}$ M and for histamine carbachol $V_{\max} = 179.4 \pm 13.2\%$ and $ED_{50} = 3.14 \pm 0.91 \cdot 10^{-4}$ M (mean \pm S.E.).

The effect of two other carbachol concentrations was also investigated in the presence of $1.1 \cdot 10^{-4}$ M histamine. Carbachol $4.5 \cdot 10^{-5}$ M increased histamine-induced respiration but not significantly above the calculated additive effect. A high concentration ($1.4 \cdot 10^{-4}$ M) gave a respiratory rate which was significantly lower than the direct additive one.

In the above expt. the rapid transient course following carbachol stimulation was encountered. This aspect was studied in kinetic expts. where the drugs were added to incubated glands. The response pattern obtained was compared with those of histamine or carbachol alone. A representative expt. is shown in Fig. 3. Histamine and carbachol follow their "normal" stimulatory courses (Berglinth *et al.* 1976) whereas the combined stimulation displayed a much steeper increase and levelled off at a steady state value which

TABLE I The effect of concomitant addition of histamine and carbachol on oxygen consumption in glands from the same populations. The table contains both the experimental and the theoretical (additive) results. The figures show mean \pm S.E. ($n=4$). The basal oxygen consumption was $12.2 \mu\text{l O}_2/\text{mg dry wt. in 90 min.}$ $p > 0.05$ is considered not significant (\pm).

Histamine		Carbachol		Histamine + carbachol $4.5 \cdot 10^{-5}$ M			
Histamine conc. (M)	Increase in oxygen consumption	Carbachol conc. (M)	Increase in oxygen consumption	Histamine conc. (M)	% Increase in oxygen consumption	Increase expected if additive	Difference found-expected
$4.2 \cdot 10^{-4}$	31.8 ± 1.3	$4.5 \cdot 10^{-5}$	9.6 ± 2.2	$4 \cdot 10^{-4}$	107.5 ± 3.7	78.3	$29.2 \pm 4.7 p$
$1.1 \cdot 10^{-3}$	84.1 ± 4.8	$4.5 \cdot 10^{-5}$	26.5 ± 2.6	$1.1 \cdot 10^{-3}$	135.3 ± 9.7	110.6	$4.6 \pm 7.5 p$
$1.1 \cdot 10^{-4}$	130.9 ± 7.8	$1.4 \cdot 10^{-4}$	30.9 ± 2.0	$1.1 \cdot 10^{-3}$	174.4 ± 12.4	157.4	$17.0 \pm 5.3 p$
Carbachol + histamine $1.1 \cdot 10^{-4}$ M							
		Carbachol conc. (M)	% Increase in oxygen consumption	Increase expected if additive	Difference found-expected		
		$4.5 \cdot 10^{-5}$	156.1 ± 11.5	140.5	15.6 ± 7.5 n.s.		
		$1.4 \cdot 10^{-4}$	143.4 ± 6.5	161.8	$-18.4 \pm 3.4 p < 0.02$		

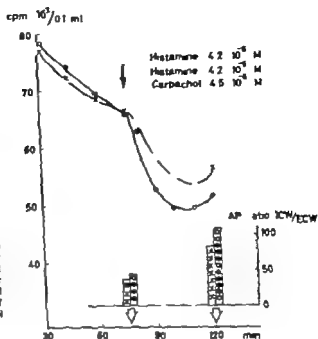


Fig. 7 The effect of histamine and carbachol on AP accumulation determined from the change in AP concentration in the basophilic fluid and the AP ratio. The latter is plotted on the left and 45 min after addition of the drugs is indicated by the bars. The concentration of drugs is 2.5 mg dry mol respectively.

In some expts. with higher aminophylline concentrations ($1 \cdot 10^{-3} \text{ M}$) a direct stimulatory influence upon accumulation was obtained. The results were, however divergent since aminophylline in these concentrations often increased the clumping of the glands (Berglin *et al.* 1976).

Histamine and carbachol. Glandular AP accumulation in these expts. was followed by determination of the AP content in the extracellular fluid. The AP ratio was measured only at certain intervals. After 30 min preincubation, AP ($0.4 \mu\text{Ci/ml}$) was added to each flask and the spontaneous accumulation studied for 45 min. Then histamine and histamine + carbachol are added in the concentrations seen in the example in Fig. 7. Following addition of the drugs, the AP uptake from the extracellular fluid was enhanced, and more so in the presence of the combined drugs than with histamine alone. This effect was also reflected in the higher AP ratio 45 min after addition.

The same experimental procedure as above for histamine and carbachol was applied to db-cAMP and carbachol.

The results indicated that carbachol may enhance the db-cAMP induced accumulation, but the effects were not as evident as for histamine.

Discussion

Previous studies on the stimulation-response coupling of the parietal cells in isolated gastric glands indicated close relationship between histamine and cAMP-stimulation (Berglin *et al.*

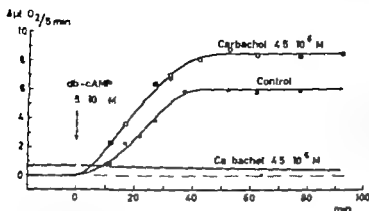


Fig. 5. The db-AMP ml. respiratory increase in glands stimulated with carbachol, let control db-cAMP is added untreated glands. The respiratory rate with carbachol alone is shown. The effect of carbachol per se (+0.2 μ l O_2 /5 min throughout subtracted from carbachol + db-cAMP curve, it facilitates exposition of the effect of carbachol on the kinetic response. The curves are drawn by eye.

the addition of carbachol produced a rapid increase in oxygen consumption up to a steady state level within 15 min. The results which are presented in Fig. 4 indicate furthermore the similarity between the histamine- and the db-cAMP responses following curial addition.

Aminopyrine accumulation

Histamine and aminophylline The influence of aminophylline on histamine-induced aminopyrine accumulation was studied by determination of the aminopyrine (AP) ratio (ICW/ECW) at each point. AP 0.4 μ Cl/ml was added to two flasks containing glands from the same population. After 45 min incubation aminophylline $5 \cdot 10^{-3}$ M was added to one flask and the effects studied for another 30 min. Histamine $5 \cdot 10^{-5}$ M was then added to both flasks and the sampling continued for a final 45 min period. The results of a typical experiment are presented in Fig. 6. The aminophylline concentrations used in these investigations did not appreciably affect the accumulation ratio, as shown in the figure. Upon addition of histamine, however, the aminophylline treated glands responded much more rapidly and reached a higher maximal accumulation than did the histamine control.

AP ratio ICW/ECW

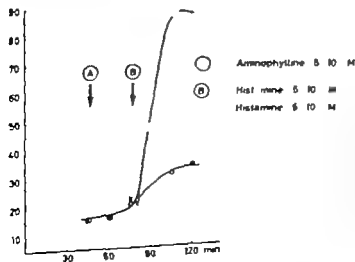


Fig. 6. Influence of aminophylline on histamine induced AP accumulation determined AP ratio (ICW/ECW). The AP was added from the start. Aminophylline is added to one flask (—) at A. The curves, however, followed the same course until histamine is added to both flask (---) at B. The concentration of gland in each flask is 7.9 mg dry wt./ml suspension.

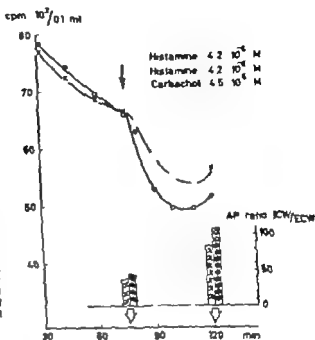


Fig. 7 The effect of histamine and carbachol on AP accumulation determined from the change in AP concentration in the extracellular fluid and the AP ratio. The latter is measured before and 45 min after addition of the drugs as indicated by the bars. The concentration of glands is 5 mg dry /ml

In some experiments with higher antidiaphyline concentrations ($1-2 \cdot 10^{-4}$ M) a direct stimulatory influence upon accumulation was obtained. The results were, however, divergent since antidiaphyline in these concentrations often increased the clumping of the glands (Berglund *et al.* 1976).

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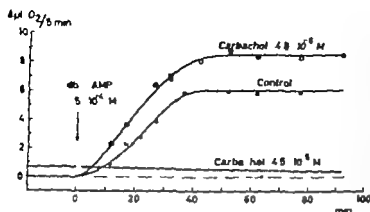


Fig. 3. The db-AMP respiratory increase in pigs stimulated with carbachol control db-cAMP was at untreated glands. The respiratory rate with carbachol alone is shown. The effect of carbachol (+0.2 μ l $O_2/5$ min) throughout subtracted in carbachol + db-cAMP can facilitate estimation of the effect of carbachol on the linear response. The curves are the

the addition of carbachol produced a rapid increase in oxygen consumption up to steady state level within 15 min. The results which are presented in Fig. 4 indicate more the similarity between the histamine- and the db-cAMP responses following carbachol addition.

Aminopyrine accumulation

Histamine and aminophylline The influence of aminophylline on histamine induced pyrine accumulation was studied by determination of the aminopyrine (AP) ratio (ICW/ECW) at each point. AP 0.4 μ Ci/ml, was added to two flasks containing glands from same population. After 45 min incubation aminophylline $5 \cdot 10^{-4}$ M was added to one and the effects studied for another 30 min. Histamine $5 \cdot 10^{-4}$ M was then added to both flasks and the sampling continued for a final 45 min period. The results of a typical experiment are presented in Fig. 6. The aminophylline concentrations used in these investigations did not appreciably affect the accumulation ratio as shown in the figure. Upon addition of histamine, however, the aminophylline treated glands responded much more rapidly and reached a higher maximal accumulation than did the histamine control.

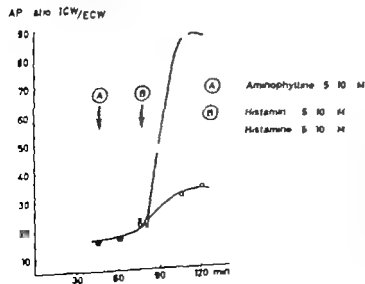


Fig. 6. Influence of aminophylline on histamine induced AP accumulation determined as AP ratio (ICW/ECW). The AP was from the start. Aminophylline was added to one flask (—) and histamine, however, followed the course until histamine was added to both flasks (---). The concentration of glands in each flask was 7.9 mg dry wt/ml suspension.

265). The desaturation effect could be abolished by a small amount of trecholine (Hirschowitz and Hutchison 1975). Thus it appears that histamine-induced secretion is normally supported by cholinergic background which might serve as a modulator of the acid secretory rate. When carbachol was added to glands prestimulated by histamine (Fig. 4), there was an almost instantaneous increase in the respiratory response up to a new steady state level. This rapid effect is in accordance with a modulator role for cholinergic stimulation. Since the simultaneous addition of histamine and carbachol induced an oxygen consumption which in fact reached its steady state later than histamine alone, a secreting parietal cell is thus a prerequisite for the rapid durable carbachol effect.

The potentiating effect also to some extent appeared to be dependent on the carbachol concentration. Thus high doses of carbachol were less effective and in fact gave values below those expected even from an additive effect alone. This is in agreement with findings *in vivo* (cf. Hirschowitz and Hutchison 1975), where the combined use of high concentrations of histamine and carbachol decreased the acid response.

Several suggestions have been put forward to explain the potentiating effect of carbachol on histamine stimulated H⁺ secretion. It has generally been proposed that cholinergic stimulation sensitizes the parietal cell, or more specifically the histamine receptor towards histamine. Recently Hirschowitz and Hutchison (1973) suggested that potentiation (increased V_{max}) between histamine and acetylcholine could be explained by postulating spare cholinergic receptors and that the synergism (increased effect of histamine) was due to an increased sensitivity of the histamine receptor. The finding in the present work that db-cAMP or histamine-induced respiratory responses were equally affected by carbachol is, however not consistent with an activation of the histamine receptor since db-cAMP exerts its stimulatory effect intracellularly.

A possible hypothesis for the potentiating effect of cholinergic stimulation might involve an influence of Ca²⁺ the importance of which has been shown for several other secretory systems. Accordingly cAMP has been shown to release Ca²⁺ from intracellular stores and acetylcholine, *in some tissues*, to increase the cell membrane permeability to Ca²⁺ and often in a transient way (for Review see Berridge 1975).

Exhorn *et al.* (1973) showed that vagal stimulation or acetylcholine infusion increased the cyclic GMP content in canine gastric mucosa in a rapid transient way. This is of interest since the only known activator of guanylate cyclase is an increase in intracellular Ca²⁺ (cf. Berridge 1975). Although the cellular localization of the cGMP increase was not determined, that result may indicate that cholinergic stimulation could be mediated by an increase in free intracellular Ca²⁺.

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- ANASTASAKIS, S. and M. J. GROSSMAN, Effect of vagal denervation of pouches on gastric secretion in dogs with intact or resected antrum. *Gastroenterology* 1963 42, 449-462.

et al 1976) The present results show that the phosphodiesterase inhibitor aminophyllin potentiates histamine-induced respiratory responses, in terms of significant increases both in ED-50 and in maximal response (V_{max}). This effect was also confirmed in the AP accumulation studies, where a potentiation of the acid secretory mechanism was revealed. The findings fulfil one of Sutherland's criteria for a hormone-cyclic AMP relationship: regard to histamine. Additional support for such a coupling is that exogenous db-cAMP (and cAMP) mimics the effects induced by histamine in the isolated gastric glands (Berglindh *et al* 1976, Berglindh 1976) and that histamine also activates adenylate cyclase from rabbit gastric mucosa (Sung *et al* 1973). It thus seems very probable that histamine-stimulate acid secretion in the rabbit is mediated by cAMP.

In *in vitro* studies on rodent gastric mucosa, an increase in cAMP content has also been shown after stimulation with cholinergic drugs or pentagastrin (Bersimbatov *et al* 1971, Narumi and Maki 1973, Rouff and Sewing 1974). *In vitro* no increase in the cAMP production with pentagastrin or carbachol in guinea pig gastric tissue was obtained (Karppanen *et al* 1974) neither did these agents stimulate adenylate cyclase from rabbit gastric mucosa (Sung *et al* 1973). The inability of aminophylline to potentiate the response to carbachol in the isolated glands are thus in agreement with other *in vitro* reports. Previously pentagastrin was shown to be without stimulating effects upon the isolated gastric glands (Berglindh *et al* 1976) and the presence of aminophylline did not alter this negative response (Berglindh unpublished results).

In a "reduced" system like isolated gastric glands only the direct response to drugs should be obtained (Berglindh and Öbrink 1976, Berglindh *et al* 1976). Since pentagastrin and cholinergic drugs stimulate the production of cAMP *in vivo* but not *in vitro* it may be suggested that they act partly via histamine, which appears equally active regardless of the type of preparation. Gastrin and pentagastrin are potent stimulators of histamine release from the mammalian gastric mucosa (*cf* Kahlson *et al* 1973) and such an effect of both pentagastrin and cholinergic stimuli has been shown in the amphibian gastric mucosa (Kasbelar *et al* 1969, Rangachari 1975). Very recently Lundell (1976) found an increase in the histamine forming capacity of the gastric mucosa in antrectomized rats following central vagal stimulation or distension of the stomach.

In vivo it is well established that histamine and cholinergic stimuli (vagal- or drug-induced) do interact and potentiate hydrochloric acid secretion (Anderson and Grossman 1965, Cooke 1969, Hirschowitz and Hutchison 1975). This relationship was also found in the isolated gastric glands, where a significant increase in oxygen consumption was recorded, above that which could be expected from an additive effect alone. In the kinetic studies when both drugs were added simultaneously the increase obtained both in oxygen consumption and in AP accumulation was more rapid and reached a higher level, than each drug alone could produce. Carbachol alone induced a transient increase in both parameters with a peak value after approximately 15 min (Berglindh *et al* 1976, Berglindh 1976) but in the presence of histamine a sustained effect was obtained. This may indicate that a "normal" cholinergic response is dependent on the presence of histamine (Berglindh *et al* 1976).

In the dog, vagal denervation of the stomach decreases its sensitivity towards histamine or pentagastrin, without altering the maximal histamine response (Andersson Grossman

(1965). The denervation effect could be abolished by a small amount of urecholine (Hirschowitz and Hutchison 1975). Thus it appears that histamine-induced secretion *in vitro* is normally supported by a cholinergic background which might serve as a modulator of the acid secretory rate. When carbachol was added to glands prestimulated by histamine (Fig. 4), there was an almost instantaneous increase in the respiratory response up to a new steady state level. This rapid effect is in accordance with a modulator role for cholinergic stimulation. Since the simultaneous addition of histamine and carbachol induced an oxygen consumption which in fact reached its steady state later than histamine alone, a secreting parietal cell is thus a prerequisite for the rapid durable carbachol effect.

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Regulation of Respiration: "The Black Box"

By

ERLING ARVENSEN

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Abstract

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A close relation between the state of wakefulness, the electroencephalogram, and the respiration has been demonstrated (Andrew 1963). In the present paper it was assumed that an opening and closing of the eyes influences the EEG and thus probably the state of wakefulness, the responsiveness of the respiratory center may likewise be influenced by the single act of closing the eyes. Seven subjects were studied in the steady state of rest breathing ambient air 4-5% CO_2 in air or 11% O_2 in nitrogen. In experimental periods of 4-5 minutes their pulmonary ventilation and alveolar Pco_2 were measured while they randomly were either reading a book, or were blindfolded. It was found that in all three conditions closing of the eyes resulted in a mean decrease of \dot{V} of 8 to 14% with a concomitant increase of Paco_2 of about 1 mmHg. It is tentatively suggested that other and stronger central or peripheral stimuli acting on the respiratory formation of the brain may explain the changed sensitivity of the respiratory center towards the chemical stimuli in conditions like exercise, chronic hypoxia etc.

In most studies of respiratory regulation, known or presumed stimuli are fed into the respiratory center (the "black box" of Defares (1964)) via the blood stream, the cerebrospinal fluid or via nervous reflex pathways, and the outcome is assumed to be the direct response to these stimuli. Several models and equations describing this relationship have been worked out (e.g. Gray (1950), Grodins *et al.* (1954), Lloyd and Cunningham (1963), Cunningham (1974)). Most of these models, however, have exemptions where they are not applicable, as for instance in muscular exercise, in sleep and during acclimatization to altitude (hypoxia). In these cases changes in the known respiratory stimuli do not elicit the predicted responses and the changes in ventilation cannot be explained as caused by these stimuli. It is an obvious thought that this may be because changes have taken place in the "black box"—the respiratory center. Such changes have earlier been described using the words "sensitivity of the respiratory center", "responsiveness" etc. (Lindhard (1911), Marius Nielsen (1936), etc.), but the expression has for several reasons fallen into discredit, partly because also sensitivity changes needed an explanation. The modern view of the respiratory center as an integrated part of the reticular formation of the brain stem with the subsequent possibilities

of influencing this part of the brain via both higher cerebral and peripheral ascending pathways provides renewed background for speaking of the sensitivity of the respiratory center. A cerebral drive responsible for the often observed continuation of ventilation after voluntary hyperventilation with below threshold P_{O_2} values, was postulated by e.g. Flink (19) and ascending proprioceptive or chemoceptive nerve impulses from exercising or moving limbs in awake man have repeatedly been ascribed the role of increasing the sensitivity of the respiratory center towards CO_2 or H^+ (e.g. Lindhard 1911, Nielsen 1936, Asmus 1973). Electroencephalography (EEG) makes it possible to get an objective signal of state of wakefulness of the brain. Bülow (1963) made extensive use of this method and demonstrated a clear correlation between various stages of EEG and respiration during wakefulness, drowsiness and sleep.

Changes in EEG are seen in awake subjects when they close their eyes (Berger 19). Supposing that this signals a changed state of arousal in the CNS, including the respiratory center, it was the purpose of the present paper to investigate possible changes in the respiratory ventilation under two conditions of awake rest: 1) resting in a reclining chair while reading a book and 2) resting in the same position but with closed eyes, blindfolded.

Methods and procedure

The pulmonary ventilation was measured as V_E from expired air collected in Douglas bags. The bags were emptied under standard low suction into a well balanced Tissot gasmeter. Samples for analysis were taken in well greased syringes and analyzed within 2 to 3 h. Inspired air was either room air, mixtures containing 4-5% CO_2 in air or 10-11% O_2 in nitrogen. The gas mixtures were administered through a large Douglas bag, maintained half filled by manipulation of the valves on the gas cylinder and containing some liters of water to moisten the air. All gases were analyzed on the Scholander apparatus. The subjects were fitted out with nose clip and breathed through a low resistance Engbøll valve. A thermometer in the valve registered the temperature fluctuations which were recorded on a ink-writing recorder at known paper speed. From the recordings the respiratory frequency was measured for use with the determination of V_E . From the ventilatory data and the gas samples the mean alveolar gas tensions could be calculated by means of the Bohr formula and a dead space estimated from the size of V_T (Asmus and Nieh 1956).

All experiments were performed in the morning in a quiet room. The subjects rested in a comfortable low chair for about 45 min. In the airbreathing experiments they then were fitted out with noseclip and mouthpiece. In the next 10-15 min were used to flush the bags with expired air and empty them again. When gas mixtures were used the gas was administered for a period of 10-25 min, including the time used to flush the bag. The actual experiment was then begun. On each experimental day 8 continuous sampling periods, each of 4 min duration were used. In 3 of these periods the subject was reading a book during the experiment. In the other three he/she was blindfolded with closed eyes. Periods with open and with closed eyes were mixed randomly and there was no pause between the periods. The number of test periods for each condition and for each subject are given in parentheses in columns 2, 3, 6, 7, 10 and 11 of Table 1.

As subjects served 5 men, aged 26 to 41 years, and 2 women, 27 and 32 years old. All were healthy, physically fit, and accustomed to respiratory procedures. One of the men knew the purpose of the study but the 6 other subjects were uninformed and did not guess the purpose.

The use of gas mixtures was first introduced because it was intended to study the effect of open or closing the eyes on the CO_2 response curves, as it was expected that potential synergistic or additive effects might be detected. As it turned out, individual and day-to-day variations of V_E and P_{ACO_2} were so large that such effects—if present—could not be seen. For the same reason the V_E with closed eyes, mean of 3 from one day will be presented as a percentage of the mean of 3 V_E with open eyes of the same day instead of absolute differences.

TABLE 1. Changes in venting respiration on closing the eyes

Subject	Breathing air				Breathing 4-5% CO ₂ in air				Breathing 11		O ₂ in nitrogen	
	V _E (STPS) l min ⁻¹		%	ΔP_{ACO_2} mmHg	V _E (STPS) l min ⁻¹		100 II/I	ΔP_{ACO_2} mmHg	V _E (STPS) l min ⁻¹		100 II/I	ΔP_{ACO_2} mmHg
	Eyes op. I	Eyes cl. II			Eyes op. I	Eyes cl. II			Eyes op. I	Eyes cl. II		
C. R. m	6.7 (6)	5.3 (8)	79	1.1	30.3 (16)	30.5 (24)	79	+0.4	—	—	—	—
T. O. I	—	—	—	—	16.2 (6)	15.6 (6)	96	+0.1	8.1 (3)	7.6 (6)	94	-0.9
B. W. I	—	—	—	—	26.8 (8)	24.4 (8)	91	+2.7	7.9* (9)	6.2 (9)	78	+4.4
H. T. m	7.6 (3)	6.8 (3)	89	+0.8	—	—	—	—	8.6 (15)	8.1 (15)	94	+0.2
G. H. m	7.8 (6)	6.4 (6)	82	0.5	25.5 (9)	22.0 (9)	86	+1.3	13.0 (18)	10.4 (9)	80	+0.2
I. R. m	—	—	—	—	30.2 (3)	24.9 (3)	82	+0.9	12.0 (9)	11.6 (9)	97	-0.3
A. B. m	12.1 (8)	11.5 (7)	95	2.7	29.8 (6)	28.3 (8)	93	+0.5	16.6 (14)	14.6 (14)	88	+0.7
Weighted means			85.4 8	+1.1			92 11	+1.1			89.6 24	+0.9
Se			1.80				1.82				2.00	

Differences between values significant on 0.025 level.

Results

Table 1 shows the average absolute values of V_E for each subject in the three different conditions of inspired air with open and with closed eyes. A Student's *t*-test performed on each individual's absolute data showed that only the data marked with an asterisk were significantly different ($p < 0.025$). This is due to the wide scattering of the day-to-day expts., a scatter which to some extent may be due to small day to day variations in the composition of the gas mixtures, when such were used as inspired air. The average percentage values (Table 1) calculated as mentioned above were also individually different and varied for the same subject from one condition to another.

The weighted means of all ratios between V_E s with closed and open eyes are also presented in Table 1, with their standard error of the mean. It can be seen that all of them are significantly different from 100.

P_{ACO_2} (Table 1) showed on an average an increase of about 1 mmHg under all three conditions when the subjects closed their eyes and V_E decreased. Individually there was 1 subject of the air expts. and 2 subjects in the low O₂-expts. who showed decrease in P_{ACO_2} . In the two latter cases the absolute values of P_{ACO_2} were about 30 mmHg and 23 mmHg, respectively—*i.e.* presumably below the threshold value for P_{CO_2} .

In spite of the 20 min pre-period when breathing gas mixtures, the nitrogen had not quite equilibrated. Accurate measurements of V_{O_2} consequently could not be made under these

conditions. But in the air expts. it was found that V_{O_2} often declined slightly when the eyes were closed. In our best studied subject (A. B.) it remained unchanged, and in his case \dot{V}_E/\dot{V}_{O_2} was found to decrease from 25.6 l/l to 24.2 l/l.

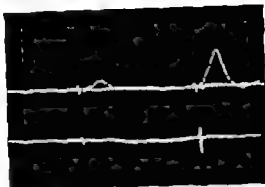
Discussion

The present expts. have shown that closing of the eyes in the resting state is accompanied by a mean reduction of V_E of 8 to 14%. These changes are small compared to the daily variations in V_E , but taken all together they are statistically significant. Also the accompanying changes in $P_{A_{CO_2}}$ (an average increase of about 1 mmHg), and in V_E/V_{O_2} (measured in air) supports the finding of a decreased V_E when the subjects have closed their eyes. The problem raised in the introduction, that the state of wakefulness or arousal has a direct effect upon the response of the respiratory center to the two stimuli P_{CO_2} (or H^+) and hypoxic P_{O_2} , thus seems to be affirmed.

The assumption that opening and closing of the eyes change the state of wakefulness based upon the well known effect on the EEG reported in the literature (Berger 1929) is supported. This change involves several autonomous and reflex functions besides the respiratory. In the present investigation it was for instance found that the briskness of a simple myoelectric reflex, the patellar reflex, was clearly correlated to the subjects' opening or closing of the eyes. In subject A. B. the average of 16 repeated standardized taps on the tendon gave a mechanical response of 1.75 arbitrary units, when the eyes were open as opposed to 0.54 when the eyes were closed. Fig. 1 shows an example with both mechanical and electromyographic responses represented. In Bülow's (1963) expts. it was clearly established that changes in the EEG from stage A (wakefulness) to stage II (drowsiness) at the beginning of sleep, was followed by changes in \dot{V}_E , and in the shape of the V_E/CO_2 response curve. The response curve shifted to the right and usually showed a less steep slope, $\Delta V_E/\Delta P_{A_{CO_2}}$. In the present expts. it was not possible to obtain CO_2 -response curves with a reproducibility sufficient to make it clear whether similar changes accompanied opening/closing of the eyes in the awake state. The average increase of about 1 mmHg in $P_{A_{CO_2}}$ with closed eyes, however, points to a right shift of the response curve, but no systematic variation in the slope of the curve was found. In hypoxia all $P_{A_{CO_2}}$ values were lowered, some of them so much (down to 23 mmHg) that the threshold value of P_{CO_2} as a respiratory stimulus had been passed. This may be one reason for the more irregular changes in $P_{A_{CO_2}}$ on closing the eyes in this condition. In the expts. show that also with hypoxia as "the sole stimulus" V_E was smaller with closed eyes.

The "change of sensitivity" of the respiratory center towards CO_2 and hypoxia demonstrated in the present expts. may well be an extreme example of what takes place when other more pronounced changes in the state of wakefulness take place. Besides sleep (for references see Bülow (1963)), morphia and other drugs are known to decrease the "sensitivity" of the center (Lindhard 1911). Bülow, whose awake state (stage A) was maintained with closed eyes, further studied a stage A_2 called "tense alertness" in which his subjects had open eyes and were alerted e.g. by having to solve problems mentally, and he actually found an increased ventilation as compared to stage A (with closed eyes). The most powerful

1. Further reflex, left: with closed eyes, right: open eyes. Top: mechanograms, middle: electrocardiogram. Subject A. B.



or for increasing the sensitivity of the respiratory center is probably muscular exercise, by acting through incoming signals from the exercising and moving limbs (Asmussen, Isen and Wæth-Pedersen (1943), Kab *et al.* (1955), Dejourn *et al.* (1964)), possibly also an increased "cortical excitatory state". The two influences—peripheral and central—will be present at the same time and the central part need not necessarily be caused by radiation of motor impulses (Krogh and Lindhard (1913)), but may come from other sensory parts of the brain. The fact that electrically induced work has the same effect (ventilation) as voluntary work (Asmussen *et al.* (1943)) thus may be explained by an raised arousal due to sensory signals from the stimulated muscles.

Nielsen (1936) showed that acclimatization to hypoxia caused a gradual increase in above the immediate chemoreceptor response to low P_{aO_2} and Forster *et al.* (1974) showed that this secondary rise in V_{O_2} was not caused by measured humoral factors in blood or CSF and suggested an increased sensitivity or responsiveness of the respiratory center caused by increased CNS arousal. This suggestion was supported by experiments with drugs (Doxapram), and with the "visual evoked response" and EEG (Forster *et al.* 1975)).

The general observation that respiration may be influenced by "the surrounding traffic" activity through the reticular formation (Burns (1963)) thus is not new. Neither is the observation that opening and closing the eyes influences the CNS state of arousal. The recent work emphasizes that respiration is not only regulated by the accepted stimuli P_{aO_2} (H^+) and P_{aCO_2} of blood or CSF but that the state of "the black box" into which these stimuli are fed may play a dominant part in what comes out of it again. Theoretically all sensory and intra-cerebral signals that impinge on the reticular formation thus may play a role in the regulation of respiration.

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Time Course of Changes in Human Skeletal Muscle Succinate Dehydrogenase and Cytochrome Oxidase Activities and Maximal Oxygen Uptake with Physical Activity and Inactivity

By

JAM HENRIKSSON and JAMES S. REITMAN¹

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Abstract

HENRIKSSON, J. and J. S. REITMAN. Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake (1) physical activity and inactivity. *Acta physiol. scand.* 1977 99 91-97

Succinate dehydrogenase (SDH) and cytochrome oxidase activities in the lateral vastus of the human quadriceps femoris muscle together with whole body \dot{V}_{O_2} max were followed during an 8-10 week period of endurance training (13) and successive 6 week period without training (8). During the training period there was gradual increases in both \dot{V}_{O_2} max and muscle oxidative enzyme activities, all being significantly different from the pre-training levels after 3 weeks of training. After 8 weeks of training \dot{V}_{O_2} max was 19%, vastus lateralis SDH 32%, and cytochrome oxidase activity 33% above the pre-training levels respectively. 6 weeks post-training \dot{V}_{O_2} max was still 16% above the pre-training level, and not significantly different from the level at the end of training ($p < 0.01$). In contrast vastus lateralis SDH activity had returned to the pre-training level. Cytochrome oxidase activity had returned to the pre-training level 4 days post-training. The significantly faster post-training decline in skeletal muscle oxidative enzyme activities in contrast to that of the \dot{V}_{O_2} max indicates that an enhancement of the oxidative potential in skeletal muscle is not necessary for high \dot{V}_{O_2} max. Moreover the fast return to the pre-training level of both SDH and cytochrome oxidase activities indicate high turnover rate of enzymes in the TCA cycle as well as the respiratory chain.

It is well documented that the maximal oxygen uptake as well as the activity of oxidative enzymes in skeletal muscles varies with the level of physical activity (for ref. see Holloszy 1975). However, there exists very little information about the time course of these changes as well as how closely they are correlated with each other.

By comparing the temporal sequence of different adaptive changes it may be possible to obtain some indications about the regulatory mechanisms of the adaptive processes. One example of this approach is given in the report by Brown *et al.* (1973), showing that during long-term electrical stimulation of a fast muscle of rabbit by frequency naturally

¹ Present address: Dept. of Internal Medicine, Bexar County Hospital, The University of Texas Health Science Center, San Antonio, Texas 78229, USA.

occurring in a nerve to a slow muscle, growth of new capillaries appears after 4 days and precedes the changes in the activity of oxidative enzymes by 10 days. This information suggests that enhanced blood supply and thus oxygen delivery to the muscle may be required for a stimulation of the synthesis of oxidative enzymes.

The purpose of the present study was to follow changes in skeletal muscle oxidative enzyme activities and relate these to the variation in maximal oxygen uptake during a period of enhanced physical activity followed by a return to normal daily activity.

Ideally all enzymes in the tricarboxylic acid (TCA) cycle and the respiratory chain should be studied. However, according to Pette and colleagues the enzyme activities within a metabolic pathway are closely related (Bass *et al.* 1969). If this holds true for humans, it is possible to limit the study to one marker enzyme representing each system. Succinate dehydrogenase (SDH) was selected as a marker for the TCA cycle and cytochrome oxidase for the respiratory chain. Data on changes in skeletal muscle capillarization and morphology will be reported separately.

Materials and Methods

13 healthy men (age 20–23 years, weight 57–110 kg, and height 160–190 cm) participated in the study. They were informed about the procedure and the risks involved in the experiments before they consented to participate. All subjects performed military service during the time of the study, their jobs consisted of office work or car-driving. None had been engaged in any regular physical training during the preceding year.

The subjects were divided into 2 groups. In the first group (group A) eight subjects were followed during a 10 week period of training (2 of these subjects continued training for an additional 4 weeks), followed by at least 6 weeks of normal physical activity at the pre-training level. An additional group of 5 subjects (group B) was followed during an 8 week training period with weekly tests within the first 3 weeks.

In group A the subjects trained by pedalling a bicycle ergometer 20 min a day at an average of 4 times a week. Work loads were initially chosen as the maximal load that the subjects could tolerate for 20 min. Work loads were increased as tolerated over the first 2 weeks but were kept constant thereafter. A crash load after 2 weeks of training corresponded to $89 \pm 10\%$ \dot{V}_{O_2} max (range 78–98%). Due to the training-induced increase in \dot{V}_{O_2} max the corresponding value at the end of the training period was 75 (range 64–84%).

In group B the training stimulus was progressively increased by continuously adjusting the mechanical work-load after measuring \dot{V}_{O_2} max so as to keep the relative work-load constant at 80% of the \dot{V}_{O_2} max. The subjects in this group were stimulated to work as long as possible during each training session by a monetary reward system. On the average these subjects trained on the bicycle 40 min a day 4 times per week. The training in both groups was supervised daily.

Muscle biopsy sampling together with \dot{V}_{O_2} max determinations were performed before as well as on repeated occasions during the training and de-training periods (Fig. 1 and 2). On testing day the subjects always came to the laboratory in the morning after a light breakfast. With few exceptions all tests and sampling were performed about 72 h after the last training session.

Maximal oxygen uptake determinations

Pre-training: The following criteria were used to ascertain that \dot{V}_{O_2} max was achieved: respiratory R value above 1, maximal heart rate and a lower measured oxygen uptake than that predicted from the work load. In group B at least 3 different \dot{V}_{O_2} max determinations were made on each subject, using work loads demanding an oxygen uptake slightly under and slightly above the measured \dot{V}_{O_2} max.

Training and de-training period: The subject always started to work at the same load that he had achieved \dot{V}_{O_2} max in the preceding test. If the subject was then estimated to be able to continue for more than 5 min at this load, the work-load was increased until the new \dot{V}_{O_2} max was reached. In this way reliable data on the changes in \dot{V}_{O_2} max during the course of the study were obtained.

Maximal oxygen uptake determinations were performed on a bicycle ergometer at 60 rpm. Inspired air

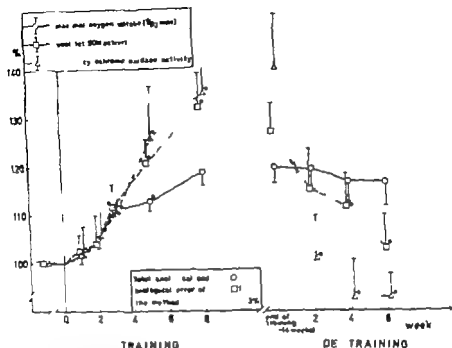


Fig. 1 Changes in V_{O_2} max and aspartate aminotransferase activity during training and de-training periods. Asterisks denote significant difference ($p < 0.05$) from pre-training (training period) or end of training (de-training period).

was collected in Douglas bags and subsequently analyzed using the Haldane technique (group A) or Beckman LB-1 infrared CO_2 analyzer and Servomex paramagnetic O_2 analyzer (OA 184) (group B). The accuracy of the latter analysis is unified with the Schoeller microtechnique.

Muscle biopsy

Before the V_{O_2} max determinations muscle biopsy (20–40 mg) is taken from the lateral area of the quadriceps femoris muscle, 12 to 16 cm above the patella, using the needle biopsy technique (Bergstrom

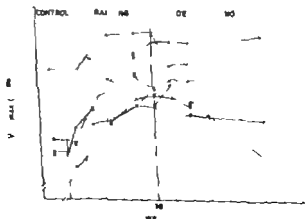


Fig. 2 Individual changes in V_{O_2} max during training and de-training periods. Solid line group A, dashed line group B.

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The subjects were divided into 2 groups. In the first group (group A) eight subjects were followed during a 10 week period of training (7 of these subjects continued training for an additional 4 weeks), followed by at least 6 weeks of normal physical activity at the pre-training level. An additional group of 5 subjects (group B) was followed during an 8 week training period, with weekly tests within the first 3 weeks.

In group A the subjects trained by pedalling a bicycle ergometer 20 min a day at an average of 4 times a week. Work-loads were initially chosen as the maximal load that the subjects could tolerate for 20 min. Work-loads were increased as tolerated over the first 2 weeks but were kept constant thereafter. A small load after 2 weeks of training corresponded to $89 \pm 6\%$ $\dot{V}_{O_{2\max}}$ (range 78-98%). Due to the training-induced increase in $\dot{V}_{O_{2\max}}$ the corresponding value at the end of the training period was 75% (range 64-84%).

In group B the training stimulus was progressively increased by continuously adjusting the work-load after measuring $\dot{V}_{O_{2\max}}$ so as to keep the relative work-load constant at 80% of the $\dot{V}_{O_{2\max}}$. The subjects in this group were stimulated to work as long as possible during each training session by monetary reward systems. On the average these subjects trained on the bicycle 40 min a day 4 times per week. The training in both groups was supervised daily.

Muscle biopsy sampling together with $\dot{V}_{O_{2\max}}$ determinations were performed before, during and after repeated occasions during the training and de-training periods (Fig. 1 and 2). On testing days the subjects always came to the laboratory in the morning after a light breakfast. With few exceptions all tests and sampling were performed about 72 h after the last training session.

Maximal oxygen uptake determinations

Pre-training: The following criteria were used to ascertain that $\dot{V}_{O_{2\max}}$ was achieved: respiratory exchange ratio ≥ 1 , maximal heart rate and lower measured oxygen uptake than that predicted from the oxygen load. In group B at least 3 different $\dot{V}_{O_{2\max}}$ determinations were made on each subject, using work-loads demanding an oxygen uptake slightly under and slightly above the measured $\dot{V}_{O_{2\max}}$.

Training and de-training periods: The subjects always started to work at the same load that had yielded $\dot{V}_{O_{2\max}}$ in the preceding rest. If the subject was then estimated to be able to continue for more than 5 min at this load, the work-load was increased until the new $\dot{V}_{O_{2\max}}$ was reached. If this was reliable data on the changes in $\dot{V}_{O_{2\max}}$ during the course of the study were obtained.

Maximal oxygen uptake determinations were performed on a bicycle ergometer at 60 rpm. Inspired air

pre-training level, but 3 subjects still had a higher \dot{V}_{O_2} max than before the training period (Fig. 2). (One subject could not be studied at this time.) The post-training decline in vastus lateralis oxidative enzyme activities revealed different time-course. 4 weeks post-training the average SDH activity was significantly lower than at the end of training ($p < 0.05$) and after further 2 weeks it had declined to the pre-training level. The cytochrome oxidase activity reached this level 2 weeks post-training, and in the following month it tended to decline even further. The de-training decline of vastus lateralis cytochrome oxidase activity expressed in percent of the value at the end of training, was significantly greater than that of \dot{V}_{O_2} max, regardless of whether it was calculated at 2 ($p < 0.001$), at 4 ($p < 0.001$) or at 6 ($p < 0.001$) weeks of de-training. A difference between the decline of \dot{V}_{O_2} max and vastus lateralis SDH activity was found 4 ($p < 0.05$) and 6 ($p < 0.001$) weeks post-training. The decline of cytochrome oxidase activity as found 2 and 4 weeks post-training was significantly greater than that of SDH ($p < 0.005$).

Discussion

During the training period no significant difference between the time-courses of change in maximal oxygen uptake and skeletal muscle oxidative enzyme activities was detected. The latter were assayed under optimal *in vitro* conditions and may be regarded as relative measures of metabolic capacities (Petts 1971). One possible interpretation of the results from the training part of the study is that the body's maximal capacity to utilize oxygen increases as a result of an enhanced oxidative potential in skeletal muscle. However the results from the de-conditioning phase show that an enhanced oxidative potential of skeletal muscle is not a necessity for an increase in total body \dot{V}_{O_2} max. Therefore, the most likely conclusion to be drawn from the present data is that exercise acts as stimulus both for factors underlying an increased \dot{V}_{O_2} max, as well as for oxidative enzyme synthesis in skeletal muscle. Thus adaptation in \dot{V}_{O_2} max is associated with but not dependent on an adaptation in skeletal muscle oxidative capacity. Accordingly discrepancies between increases in muscle oxidative capacity and \dot{V}_{O_2} max have been reported (Gollnick *et al.* 1973, Henriksson and Rasmussen 1976). Increased muscle oxidative capacity may be more important during submaximal work, contributing to a shift towards greater fat relative to carbohydrate metabolism (Holloszy 1967, Karlsson *et al.* 1974).

The metabolic capacities of the TCA cycle and of the respiratory chain were increased to the same extent during the training period, which confirms earlier reports based on animal studies (Holloszy 1975). The de-training decline in activity of the respiratory chain marker cytochrome oxidase was significantly faster than that of the TCA cycle marker SDH. This is somewhat surprising in view of the fact that both enzymes are situated on the inner mitochondrial membrane and therefore might be expected to turn over with similar half-lives (Rabinowitz and Zak 1975). If an exponential curve is fitted for the decline in enzyme activity the half-life of the enzymes can be calculated using a first-order kinetic model as described by Schmitke and Doyle (1970). Determined in this way the half-life of cytochrome oxidase is 5 days. In view of the limited number of muscle samples analyzed in the de-training period this value is to be considered as an estimate. However it is of the same order of magnitude as reported for cytochrome C turnover in rat muscle, where the calculated

1962). In group B two samples were obtained before the training period, one before and the other 4-6 days before the 3-4 days of \dot{V}_{O_2} max determinations. As these two samples did not differ significantly in either SDH or cytochrome oxidase activities, the averages were used as pre-training values for group B. Up to five biopsies were taken from the same subject in group A, alternating between the left and the right leg; in group B all biopsies were taken from the same leg.

The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent enzyme analysis. The enzyme analyses were performed within 2-3 months.

For SDH analysis the muscle was homogenized in 0.3 M phosphate buffer (pH 7.7) with 0.05 M and analyzed as described previously (Essén *et al.* 1973).

For cytochrome oxidase analysis the muscle was homogenized in 0.01 M phosphate buffer (pH 7.5) with 0.05% BSA. From the homogenate 100 μl was added to 1 ml of a stirred 0.05 M phosphate buffer (pH 7.5). The reaction mixture which was kept at 30°C , was then made 30 mM with respect to N,N,N',N'-tetramethylphenylenediamine-dihydrochloride (TMPD), 50 mM with respect to sodium ascorbate and 10 mM with respect to cytochrome C. The O_2 consumption was then followed with a Clark oxygen electrode. After subtracting the oxygen consumption of a blank solution, where the muscle homogenate had been replaced by the same volume of homogenizing medium, the cytochrome oxidase activity could be calculated.

In all subjects tests were made to determine whether enzyme activities were different in the 2 legs. In such differences were found in either SDH or cytochrome oxidase activities. SD of the difference between the right and the left leg was 1.5 (SDH) and 0.9 $\mu\text{mol g}^{-1}$ (wet weight) min^{-1} (cytochrome oxidase) in both cases, 14% of the mean value. This variability was of the same order of magnitude as that seen when a biopsy was taken from the same vastus lateralis on different days. SD of the difference between 13% (SDH $n=18$) and 14% (cytochrome oxidase, $n=8$) of the mean value.

Statistics. The significance of intrasubject differences was tested using Student's *t*-test.

Results

In spite of the different design of the training programmes in the 2 groups, no significant differences in changes of either \dot{V}_{O_2} max or enzyme activities were seen at any time. Therefore, in the following, the 2 groups will be considered together. All results are presented as per cent change from the pre-training level which for \dot{V}_{O_2} max was 3.27 l min^{-1} for SDH and cytochrome oxidase activities 9.3 and 7.1 $\mu\text{mol g}^{-1}$ (wet weight) min^{-1} respectively.

During the training \dot{V}_{O_2} max and vastus lateralis oxidative enzyme activities increased gradually and were significantly different from the pre-training levels after 3 weeks (Fig. 1). At this time the average \dot{V}_{O_2} max was 11.1% above the pre-training level ($p < 0.001$). Vastus lateralis SDH and cytochrome oxidase activities were then 11.5% ($p < 0.05$) and 11.0% ($p < 0.05$) above the pre-training levels, respectively. After 5 and 8 weeks of training \dot{V}_{O_2} max was 12.6% ($p < 0.001$) and 18.6% ($p < 0.001$) higher than the pre-training level. Corresponding values for SDH and cytochrome oxidase activities were, after 5 weeks, 20.5% ($p < 0.005$) and 25.6% ($p < 0.05$), and after 8 weeks, 32.0% ($p < 0.001$) and 34.7% ($p < 0.005$), respectively. Both after 5 and 8 weeks of training the average increase in muscle oxidative enzyme activities was considerably greater than that of \dot{V}_{O_2} max, however the differences were at neither occasion statistically significant.

De-training period. Of the 8 subjects (group A) who were studied in this period 6 discontinued training after 10 and two after 14 weeks. The results at these intervals are indicated as end of training values in Fig. 1.

The average \dot{V}_{O_2} max at 6 weeks post-training was still 16% higher than the pre-training value ($p < 0.01$) and not significantly different from the value at end of the training ($p > 0.2$). 12 weeks after cessation of training \dot{V}_{O_2} max in 4 out of the 8 subjects had returned to the

training level, but 3 subjects still had a higher V_{O_2} max than before the training period. 2). (One subject could not be studied at this time.) The post-training decline in vastus lateralis oxidative enzyme activities revealed a different time-course. 4 weeks post-training average SDH activity was significantly lower than at the end of training ($p < 0.05$) after further 2 weeks it had declined to the pre-training level. The cytochrome oxidase very reached this level 2 weeks post-training, and in the following month it tended to increase even further. The de-training decline of vastus lateralis cytochrome oxidase activity expressed in percent of the value at the end of training, was significantly greater than that of V_{O_2} max, regardless of whether it was calculated at 2 ($p < 0.001$), at 4 ($p < 0.001$) or at 6 ($p < 0.001$) weeks of de-training. A difference between the decline of V_{O_2} max and vastus lateralis SDH activity was found 4 ($p < 0.05$) and 6 ($p < 0.001$) weeks post-training. The decline of cytochrome oxidase activity as found 2 and 4 weeks post-training was significantly steeper than that of SDH ($p < 0.005$).

Discussion

During the training period no significant difference between the time-courses of change in maximal oxygen uptake and skeletal muscle oxidative enzyme activities was detected. The latter were assayed under optimal *in vitro* conditions and may be regarded as relative measures of metabolic capacities (Petto 1971). One possible interpretation of the results from the training part of the study is that the body's maximal capacity to utilize oxygen increases as a result of an enhanced oxidative potential in skeletal muscle. However the results from the de-conditioning phase show that an enhanced oxidative potential of skeletal muscle is not a necessary for an increase in total body V_{O_2} max. Therefore, the most likely conclusion to be drawn from the present data is that exercise acts as stimulus both for factors underlying an increased V_{O_2} max, as well as for oxidative enzyme synthesis in skeletal muscle. Thus adaptation in V_{O_2} max is associated with but not dependent on an adaptation in skeletal muscle oxidative capacity. Accordingly discrepancies between increases in muscle oxidative capacity and V_{O_2} max have been reported (Gollnick *et al.* 1973, Hermanson and Reitman 1976). Increased muscle oxidative capacity may be more important during submaximal work, contributing to a shift towards a greater fat relative to carbohydrate metabolism (Holloszy 1967, Karlsson *et al.* 1974).

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half life was 8 days (Terjung 1975). Due to the scatter around the curve, the same type calculation for SDH activity is more uncertain, but the half-life obtained is at least 2 times longer than that of cytochrome oxidase.

If the degradation rate of mitochondrial enzymes is of the magnitude indicated by the half life of cytochrome oxidase, this might explain the relatively low percentage loss in skeletal muscle oxidative capacity normally seen in humans with training programs of the same type as in the present study. De-training effects between exercise bouts influence the rise in oxidative enzyme activities during the training period, making it less than it would theoretically be if a maximal training stimulus could have been imposed. With a gradually increasing stimulus over a sufficient period of time the oxidative capacity of human skeletal muscle can increase considerably. Gollnick *et al.* (1973) for example observed a doubling in vastus lateralis SDH activity after 5 months of extensive endurance training.

No significant differences in the magnitude and time-course of the adaptive change between the two training groups were found. However, between the fifth and the eighth week of training there was a tendency toward a continuous increase in SDH and cytochrome oxidase activities (20 and 17 per cent) in group II, whereas in group I these appeared to have levelled off after the fifth training week. These findings are then in accordance with the results of Benzi *et al.* (1975), who found in rats, that in response to a constant training stimulus the oxidative enzyme activities tended to level off at a certain level, which they changed if a new level of physical activity was imposed on the animals.

The results of the present study indicate, that with changes in the physical activity level fast adaptive changes occur both at the local muscle and the systemic level in the maximal capacity to utilize oxygen. The discrepancies in post training decline of maximal oxygen uptake and leg muscle oxidative capacity indicate that the latter is not the limiting factor determining the body's maximal capacity to utilize oxygen. During the training period this is further substantiated by the tendency towards a greater percentage increase in skeletal muscle oxidative enzyme activities as opposed to maximal oxygen uptake.

This study was made possible by grants from the Danish Natural Science Research Council and the Research Council of the Danish Sports Federation.

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The Effect of Plasma Proteins on the Capillary Permeability in the *rete mirabile* of the Eel (*Anguilla vulgaris* L.)

By

KJELL MYHRE and JOHAN B. STEEN

Received 14 July 1976

Abstract

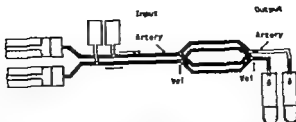
MYHRE, KJ and J. B. STEEN. The effect of plasma proteins on the capillary permeability in the *rete mirabile* of the eel (*Anguilla vulgaris* L.). Acta physiol. scand. 1977 99 98-104.

Measurements of transcapillary exchange of high-molecular dextrans, K^+ and THO in the *rete mirabile* of the eel (*Anguilla vulgaris* L.) have been made under steady-state conditions while varying the amount of plasma proteins in the perfusates. When the perfusates contained 20% or less horse serum the permeabilities of D-75 000, K^+ and THO increased significantly. The effect on the K^+ permeability was reversible while that on D-75 000 and THO was not. The permeability was unaffected when the perfusate contained 30% serum or more. Adding 1.5 g/100 ml bovine albumin to the perfusate maintained the D-75 000 and THO permeabilities unchanged while that of K^+ appeared to increase. At an albumin content of 1 g/100 ml the effect was similar to that produced by low serum perfusates. Our main conclusion is that 1% of the albumin fraction of the plasma proteins reversibly increases the interstitial pore area and irreversibly changes the K^+ permeability of the cell membrane.

Krogh and Harrop (1921) demonstrated that when the leg of the common frog (*Rana temporaria*) was perfused with a Ringer solution containing 3% gum-acacia, the capillaries dilated and the tissue became edematous unless defibrinated ox blood was added to the perfusate. Since then several blood constituents have been shown to participate in the maintenance of normal capillary permeability in the frog. According to Drinker (1927) the addition of 15% to 20% horse serum prevented edema. Saslow (1938) however claimed that red cells had to be present, while Danielli (1940) showed that both red cells and platelets retarded the development of edema. Recently bovine albumin has been shown to reduce the mesenteric capillary permeability of T 1824 in the frog (Levick and Michel 1973 a, b).

Danielli (1940) suggested that the platelets blocked the protein-permeable pores, while Landis and Pappenheimer (1963) proposed that the plasma proteins combined loosely with the walls of the capillary pores and reduced their cross-sectional area. The latter view has been supported by Levick and Michel (1973 b).

As previously shown by Stray-Pedersen and Steen (1975) and Myhre, Steen and Stray-Pedersen (1976) the *rete mirabile* in the swimbladder of the common eel is useful in studying capillary permeability. It consists of parallel arterial and venous capillaries running close together as first described by Krogh (1929).



Schematic drawing of the experimental design. The perfusates are delivered to the *rete* from 2 in the input artery and vein. The syringes (A and V) are located in perfusion pump and the syringe (A) contained the test substances. The input pressures were measured by its pressure transducer (P) connected to the input tubes by T-couplings. The outflow from the *rete* was collected from its artery and vein in two pre-weighed test tubes.

In present expts the *rete* of the eel was perfused with a Ringer Dextran solution while the effect on the permeability of Dextran-75 000, K and THO was evaluated at varying ratios of protein in the perfusates.

Material and methods

Eels, weighing 200 g to 400 g, were caught in sea water and kept in running fresh water in the laboratory.

Preparation of the eel. After making the fish unconscious by blow on the head the swimbladder was removed and eel was mounted on a table top. The artery and vein on the heart side of the *rete* (in the present study input artery and vein, Fig. 1) were cannulated with polyethylene tubes (Lectromedic, R, PE 60 and 10) and small glass tubes were inserted in an artery/vein pair on the bladder side of the *rete* (output artery and vein). Broad-plated bulldog clamps were used to close the numerous vessels branching in the output artery and vein.

Perfusion. The *rete* was perfused in parallel fashion. The input tubes were connected to two glass-syringes driven by a Perfusion Pump (Braun-Melton-Gum) accuracy $\pm 1\%$ of nominal flow. The perfusates from the pump were collected in pre-weighed test tubes. The rate of output flow was calculated from the weight of the test tubes and the time of collecting. At the start of the expts the output flow (0.2 ml/min–0.5 ml/min) was adjusted to give perfusion pressures of 30–50 cm H₂O in the input artery and 10–15 cm H₂O in the input vein to expose the *rete* to near normal conditions (Mott 1930), and maintained constant throughout the expts. The input arterial-venous pressure difference was not allowed to change more than ± 2 cm H₂O at discharging the expts. The output pressures were measured by two Statham P23 De transducers connected to the output tubes by side branches (Fig. 1), and continuously registered on a Beckman Dynagraph R3. Further details are described in Mylre, Sævi and Eide-Pedersen (1976).

Human serum. Human serum was obtained commercially (Gibco Bio-Cult, Glasgow, Scotland) and stored at -20°C prior to the expts. The serum was thawed at room temperature and filtered.

Ringer Dextran solution. The solution contained 130 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 2 mM H₂O, 5.6 mM glucose and 0.43 mM Dextran T 70 (MW 70 000). All chemicals were of analytical grade. The osmolality of the solution was about 290 mOsmol/kg. It matched approximately eel plasma concerning the osmotic pressure. The Ca^{2+} content was doubled, and made possible 4 to 5 h perfusion of the *rete* at near normal stable conditions.

Human plasma. In some experiments human albumin (Armour Pharmaceutical Company Chicago Ill. U.S.A.) was added to the Ringer-Dextran solution in concentrations 1.3 g/100 ml or 3.3 g/100 ml. This corresponds to 10 and 30 respectively of that present in human plasma.

Heparin. Heparin and frozen human plasma (20 LU Heparin A L-fred) was prior to an expt, thawed at room temperature and filtered.

Perfusates. The perfusates were equilibrated with air prior to the expts. Papaverine sulphate (0.2 mg/ml) was added to the perfusates in order to secure stable perfusion conditions (Eide-Pedersen and Sævi 1975). This solution was crystallized, however at pH above 6.0. Consequently the pH of all perfusates was adjusted to 5.9 with NaOH or HCl.

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TABLE 1. Average permeabilities of D-75 000, K, and THO, standard errors of the mean and range of transcapillary liquid pressures in the initial phase of the experiments.

molecule	Range of transcapillary liquid pressures (cm H ₂ O)	Average permeability area (PS) \pm S.E. (10^{-3} cm ² s ⁻¹)		
		D-75 000	K	THO
1	10-42	2.29 \pm 0.26	3.97 \pm 0.51	97.89 \pm 15.23

Results

Table 1 the permeabilities of D-75 000, K, and THO in the initial phase are listed as the mean value of all expts. THO penetrated the capillary walls about 24 times as fast as K, which in turn penetrated about 2 times faster than D-75 000.

Fig. 2 illustrates the effect of serum on the vascular bed. The capillary permeabilities of the test molecules obtained with perfusates containing 50% (Group A) and 30% (Group B) horse serum did not differ from those obtained by 100% serum. With lower serum content in the perfusates (Group C, F) the permeability of D-75 000, K, and THO increased. Perfusates consisting of 100% Ringer-Dextran (Group F) did not give a significantly different response from that obtained when the test was perfused with 80% Ringer Dextran and

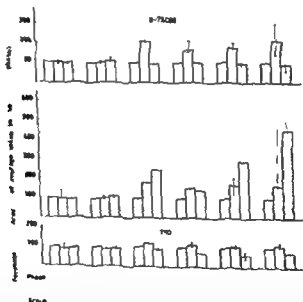


Fig. 2. The effect on the permeabilities of D-75 000, K, and THO of different concentrations of serum in the perfusates. In the initial (I) and recovery (II) phases the test was perfused with 100% serum while the serum in the experimental phases (III-VI) was diluted with Ringer-Dextran. The experiments were separated in groups according to the content of serum in the perfusates. (Group A, 50% serum; Group B, 30% serum; Group C, 20% serum; Group D, 10% serum; Group E, 5% serum; Group F, 0% serum.) The permeabilities for D-75 000 and THO in the experimental phases are averaged from all samples collected, but for K, from the last 3 samples only. The permeabilities are expressed in per cent of the average permeability in the initial phase and plotted as the median of N expts. The vertical bars mark the range of the permeabilities in different expts.

Transcapillary gradients were obtained by adding K^+ (as KCl), tritium labelled after T110 (Kjeller Norway) and Dextran-Carboxyl- 4C (D-75 000), MW 60 000-90 000 (NEC 218 B, New England Nuclear Mass. U.S.A.) to the arterial perfusates. Equimolar amounts of NaCl were added to the venous perfusate to maintain isotonic conditions.

Experiments. 3 series of expts. were carried out. In the first series we compared the capillary permeabilities of the test substances during serum perfusion to the permeabilities obtained when serum was diluted with Ringer-Dextran. The perfusates contained 100%, 50%, 30%, 20%, 10%, 1% and 0% horse serum.

Since perfusates containing less than 20% serum caused typical vascular changes, we tested in the second series the effect of bovine albumin. To this end we compared the permeabilities during serum perfusion to those during perfusion with Ringer-Dextran containing albumin. Two concentrations of bovine albumin were used. The first corresponded to the content of albumin in a perfusate of 40% horse serum in Ringer-Dextran (1.3 g/100 ml), the second concentration to 10% serum in Ringer-Dextran (0.33 g/100 ml).

To test for possible effects of substances removed by defibrination of plasma, we compared, in the third series, the permeabilities during serum perfusion with those obtained during horse plasma perfusion.

Each expt. was designed in a standard way. It started with an initial phase of serum perfusion. When stable permeability values had been obtained, the rete was, in the experimental phase, perfused with the test perfusate. The expt. was terminated with a recovery phase of serum perfusion.

The initial phase lasted 30-60 min and 3-4 samples of the outflow were collected. The experimental phase lasted 25-100 min and at least 3 samples were collected. The recovery phase was terminated when the transcapillary input pressures changed more than ± 1 cm H_2O and as many samples as possible were collected.

The experiments were terminated by perfusing the rete with a filtered solution of Indian ink to determine the location of leakages. Leakages in the input vessels or in the rete would reduce the flow through the capillaries and when this occurred the expt. was discarded.

Analysis. The concentrations of the test substances were measured in the input and output perfusates. Samples of the input perfusates were collected at the beginning and end of each phase and analysed in triplicate, while the output samples were analysed in duplicate.

K: The concentrations of K were measured with a flame photometer (Flame Photometer 34, Instrumentation Laboratory (U.K.) Ltd.). Accuracy was within $\pm 2\%$ in the actual range of concentration (3-9 mEq/l).

THO Dextran-Carboxyl- 4C : The Packard Tri-Carb Liquid Scintillation Spectrometer Model 130 was used to measure the concentrations of the radioactive labelled test substances. To achieve adequate counting accuracy the activities in the input perfusates were adjusted to obtain at least 10 000 counts in the outflow samples. Instagel (Packard Instrument Co.) was used as scintillation liquid.

Calculations. The permeabilities of the test substances were calculated from steady-state retinal flow and concentrations measured in the input and output perfusates. The term permeability as it is used in this study refers to total exchanges either carried by bulk flow or by diffusion. Bulk flow will be most important for Dextran, diffusion for K and especially for THO (Myhre, Steen and Stray-Pedersen 1976). The total available for transcapillary exchange in the rete was not measured. Hence the permeability (PS) is expressed as the product of the permeability coefficient (P) and the area (S), according to (1)

$$PS = \frac{1}{2} V_f \frac{\Delta C_a}{\Delta C_v} \quad (1)$$

V_f = arterial (venous) flow

ΔC_a = arterio-venous concentration difference of the test substances in the input perfusates

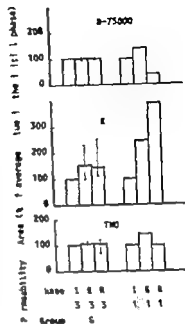
ΔC_v = arterio-venous concentration difference of the test substances in the output perfusates

The concentrations of K^+ in the output perfusates will be affected by contamination of intracellular potassium. If we assume that the arterial and venous perfusate receives approximately the same amount of potassium from the tissue, the permeability calculated according to (1) will not be affected. An imbalance will, however, appear between the apparent K gain in the venous perfusate and the K loss from the arterial perfusate.

Both analytical procedures and the transcapillary exchange will influence the accuracy of the permeabilities. Consequently it is got from about 5% to about 10% with the highest accuracy for the most permeable substances.

The results were treated statistically with the Wilcoxon Matched-pairs Signed-ranks test (Siegel 1956, p. 75).

Fig. 4 The effect of albumin in the perfusate on the permeability of D-75 000, K, and THO. In Group II the Ringer-Dextran perfusate contained 1.3 g/100 ml bovine albumin, in Group III a solution content was 0.13 g/100 ml. The permeabilities are given as per cent of the average permeability of the control and plotted as the means of 4 experiments. The vertical bars denote the range of the permeability. Abbreviations as in Fig. 3.



When the rete was perfused with horse plasma the capillary permeability did not differ from the values obtained when the perfusate contained 100% serum.

Discussion

The present study demonstrated that the capillary permeability of the eel rete increased when the perfusate contained less than 30% horse serum, or bovine albumin corresponding to less than 40% serum. Krogh and Harrop (1921) did similar observations on frog muscles perfused with Ringer solution containing 3% gum-acacia and supplemented with ox serum. It is therefore suggested that the albumin fraction of plasma takes part in maintaining normal capillary permeability in the rete.

Lack of serum in the perfusate affected the permeability of K differently from that of D-75 000 and THO. Contrary to D-75 000 and THO the K permeability increased progressively during perfusion with low serum perfusates. The effect on D-75 000 and THO must reside in the intercellular pores since it was readily reversed. The effect on K was not reversible and resides most likely in the K⁺-pores of the endothelial cell membranes. This conclusion is supported by the observed leakage of K⁺ from the retial tissue during perfusion with solutions containing 20% or less serum.

Adding 1.3 g/100 ml bovine albumin gave a certain increase of the K permeability. Aside from the fact that the albumin was bovine, this observation might indicate that either higher albumin levels or some other serum fraction was needed to stabilize the cell membranes. In this connection one should note that the experiments of Stray-Pedersen (1971) indicated that the protein level which is necessary to maintain normal permeability con-

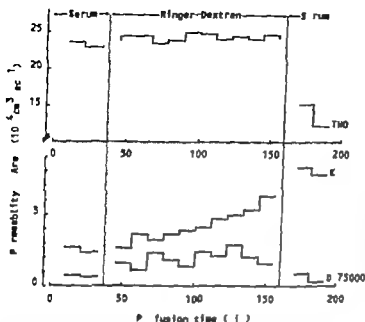


Fig. 3. The effect on the permeabilities (PS) ($10^{-4} \text{ cm}^3 \text{ sec}^{-1}$) of D-75 000, K, and THO when the *rete* was perfused with a Ringer-Dextran solution. The transcapillary input pressure remained constant throughout the expt.

20% horse serum. The permeabilities obtained during perfusion with less than 20% horse serum in the perfusate were significantly higher than those obtained during serum perfusion ($p < 0.002$ for Groups C, D, E and $p < 0.005$ for Group F).

During perfusion with less than 30% serum there was clear evidence of leakage of potassium from the retial tissue. This leakage was manifested as an apparently larger gain in venous K^+ concentration than should be expected according to the decline in arterial K^+ concentration. Analysis of cellular ionic changes was not performed.

The increased permeability of D-75 000 and THO produced by low serum concentrations in the perfusate could be restored to normal by perfusing the *rete* with horse serum. In contrast, the increased K^+ permeability could not be reversed. Perfusates with low serum contents also produced other signs of major vascular changes. In several experiments the input pressures started to increase and the *rete* appeared edematous after about 30 min of perfusion with no detectable vascular changes. This is the reason why the number of recovery expts. is reduced in several expts.

The change in permeability of the three test substances progressed differently during perfusions with low serum solutions. The K^+ permeability was increased from the first sample and continued to increase throughout the perfusion period. The permeabilities of D-75 000 and THO however increased initially and were maintained at fairly stable levels throughout the experimental phase. Fig. 3 shows results from a typical expt. in which the perfusate was 100% Ringer-Dextran.

Fig. 4 shows the effect of bovine albumin. No change in the permeabilities of D-75 000 and THO was observed when the Ringer-Dextran solution contained 1.3 g/100 ml albumin, whereas the albumin concentration of 0.33 g/100 ml produced the same effect as when the Ringer-Dextran perfusate contained 20% or less serum. The K^+ permeability increased slightly but statistically not significantly at an albumin concentration of 1.3 g/100 ml, but markedly when the concentration was 0.33 g/100 ml. The K^+ permeability did not return to normal in the recovery phase.

Skeletal Muscle Fiber Splitting Induced by Weight Lifting Exercise in Cats

By

W. GONYEA, G. C. ERICSON and F. BONDE-PETERSEN

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Abstract

GONYEA, W., ERICSON, G. C. and BONDE-PETERSEN, F. Skeletal muscle fiber splitting induced by weight-lifting exercise in cats. *Acta physiol. scand.* 1977 99 105-109

Skeletal muscle hypertrophy induced by exercise has been thought to be exclusively related to an increase in cross-section area of individual muscle fibers and not to an increase in the number of muscle fibers. Recent experiments using surgical intervention to cause muscle overload have induced an increase in fiber numbers, however, the muscle also exhibited pathological alterations. The purpose of this study was to determine if an exercise regimen also induced hyperplasia. Cats were trained to lift weights with right forelimbs to receive food reward. After 19-46 weeks of training, the flexor carpi radialis muscle (FCR) was removed and prepared for histochemical examination. The total number of muscle fibers in right exercised FCR increased significantly (19.3%) when compared with that of the unexercised FCR ($p < 0.05$). This increase was found to be due to muscle fiber splitting.

Since the work of Morpurgo (1897), the concept has been prevailing that skeletal muscle hypertrophy after training is exclusively related to increase in cross-section area of individual muscle fibers while the total number of fibers in a given muscle remains unaffected by training.

However recent observations indicate that an increase in the number of muscle fibers (hyperplasia) as well as increase in cross-section area (hypertrophy) of the individual fibers can be induced in muscles trained after removal or denervation of synergistic muscles or after endurance training (VanLinge 1962, Edgerton 1970, Hall-Craggs 1970, Reitzner 1970, Jones 1973, Soia *et al.* 1973). The histological picture of these overworked muscles also shows degenerative changes as e.g. hyaline degeneration, karyopyknosis and monocyte infiltration. It seems, therefore, of importance to establish experimental evidence whether the reported hyperplasia also occurs in training of unrestrained animals during weight lifting exercise. Such an investigation was the aim of the present study.

tions may well be different at higher perfusate pH and vary with the ionic composition of the perfusate.

The observed ability of serum proteins to maintain normal permeability in the rete reports similar observations in the frog (Drinkier 1927 Saslow 1938, Danielli 1940, Lea and Michel 1973 a, b Mason, Michel and Tooke 1973). This effect seems not to be related to the osmotic activity of the plasma proteins, since the rete vessels were perfused with isosmotic solutions. The increased capillary permeability cannot be produced by adequate oxygenation of the perfusate, as claimed by Saslow (1938), since all the perfusates in the present study were equilibrated with air.

Contrary to Danielli (1940) who proposed that the intercellular pores were specifically blocked by platelets, Lardis and Pappenheimer (1963) suggested that the intercellular pore area was normally reduced by adsorbed plasma proteins. Their view was supported by Levick and Michel (1973 a, b) who reported that bovine albumin halved the permeability of T 1824 in the mesenteric capillaries of frogs. The results of the present study represent further support of the view that the albumin fraction of the plasma proteins reduces the cross-sectional area of the intercellular pores.

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However, recent observations indicate that an increase in the number of muscle fibers (hyperplasia) as well as increase in cross-section area (hypertrophy) of the individual fibers can be induced in muscles trained after removal or denervation of synergistic muscles or after endurance training (VanLinge 1962, Edgerton 1970, Hall-Craggs 1970, Reisman 1970, James 1973, Sola *et al.* 1973). The histological picture of these overworked muscles also shows degenerative changes as e.g. hyaline degeneration, karyopyknosis and monocyte infiltration. It seems, therefore, of importance to establish experimental evidence whether the reported hyperplasia also occurs in training of unrestrained animals during weight lifting exercise. Such an investigation was the aim of the present study.

tions may well be different at higher perfusate pH and vary with the ionic composition of the perfusate.

The observed ability of serum proteins to maintain normal permeability in the *rete* ports similar observations in the frog (Drinker 1927 Saslow 1938, Danielli 1940, Li and Michel 1973 a, b, Mason, Michel and Tooko 1973). This effect seems not related to the osmotic activity of the plasma proteins, since the *rete* vessels were perfused with isosmotic solutions. The increased capillary permeability cannot be produced by inadequate oxygenation of the perfusate, as claimed by Saslow (1938), since all the perfusates in the present study were equilibrated with air.

Contrary to Danielli (1940) who proposed that the intercellular pores were blocked by platelets, Lardis and Pappenheimer (1963) suggested that the intercellular area was normally reduced by adsorbed plasma proteins. Their view was supported by Vick and Michel (1973 a, b) who reported that bovine albumin halved the permeability of T 1824 in the mesenteric capillaries of frogs. The results of the present study represent further support of the view that the albumin fraction of the plasma proteins reduces the cross-sectional area of the intercellular pores.

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TABLE I. Mean values and S.E. for measured and calculated characteristics of the flexor carpi radialis muscle for control and exercised cats.

		Control cats (N=10)			Exercised cats (N=5)		
		Left limb	Right limb	difference	Left limb	Right limb	difference
(10)	\bar{x} S.E.	1.34 (0.136)	1.32 (0.133)	-1.49	1.34 (0.201)	1.43 (0.220)	6.72*
total	\bar{x} S.E.	8799 (263)	9001 (243)	2.30	7809 [7701] [8086] [7839]	9081 [10202] [8854] [8185]	19.3
SO	\bar{x} S.E.	37.1 (1.60)	35.6 (1.39)	-1.50	43.6 (4.17)	37.4 (1.49)	-6.20
FOG	\bar{x} S.E.	25.2 (3.11)	28.1 (2.73)	2.90	27.8 (4.30)	37.0 (4.81)	9.20
FG	\bar{x} S.E.	37.7 (2.78)	36.4 (3.39)	1.30	28.6 (2.14)	25.5 (3.44)	-3.10

(Controls = 6, exercised = 3). *Ousted to be significant $p < 0.05$; [3 measurements for exercised cats]

the right limb of the total fiber count in the control animals, and the differences between limbs were not significant. In the trained animals, the exercised FCR showed 37.4 SO fibers compared with 43.6% on the nonexercised side. However these differences were not significant, nor did these proportions differ significantly from those of the control animals. In addition, the distribution of FOG and FG fibers showed no significant differences between the left and right muscles of the control and trained animals (Table I). The lack of significant differences in the relative fiber content of the left and right FCR from trained animals could indicate that the absolute increase in total muscle fibers in the exercised FCR is due to a parallel increase in both fast and slow-twitch fibers. However there was a substantial 9.20%, although not significant increase in FOG fibers. The lack of significance is probably due to small sample size (3) with a large variance. This finding is consistent with the observation that all fiber types undergo significant hypertrophy during weight-lifting exercise, however the increase is greater for the FOG fibers (SO, 10.1%, FG 10.8%, FOG 15.1%, Gonyea and Ericson 1976).

The histological picture showed fiber splitting of both fast-twitch and slow-twitch fibers in the trained muscles (Fig. 1). Even by careful examination of the serial sections, which virtually comprised the total length of the FCR, it was not possible to observe fiber splitting in the control animals. A few fibers were also observed to be splitting in the left FCR of the trained animals only. Even though the left limb is not directly exercised, it is used as a supportive brace during training (Gonyea and Ericson 1976). This finding has the effect of making it more difficult to demonstrate significant differences when the total number of muscle fibers was compared between limbs. The data indicates that one possible mechanism for the significant increase in the total number of fibers in the exercised FCR is due to fiber splitting. Whether satellite cells are also involved in this process as has been postulated by several investigators (Schiaffino *et al.* 1972, James 1973) is not known at this time. To

Methods

A control group of 10 adult cats (body wt. 3.70 ± 0.204 kg) and a training group of 5 adult cats (body wt. 3.57 ± 0.164 kg) were established. The control animals were confined to individual cages for a period of 4-8 weeks. The weight-lifting exercise training was carried out according to Gonyea and Ericson (1971). The cats were conditioned to move a bar a specific distance with their right forelimb to receive a reward. Weights attached to the bar via a pulley were lifted as the bar was moved. During the last 4 weeks of training, the animals lifted 100 g and at the end of 19-46 weeks of training, they lifted 1.12 kg (mean \pm S.E.). In this preliminary study the animals were not exercised until heavier weights could no longer be lifted. Therefore the data must be considered as giving only an indication of the degree of hypertrophy that can be expected from cats that are exercised at heavier loads.

On the day of investigation, the *m. flexor carpi radialis* (FCR) was dissected during Nembutal anaesthesia and weighed. The cat was then sacrificed with an overdose of Nembutal and the remaining 37 muscle groups of both anterior limbs were removed and weighed. The procedures outlined by La (1917) in obtaining muscle specimens were adhered to in this study. In this regard, the muscles were freed from their attachments by sub-periosteal incision and cleaned of all extraneous connective tissue. All tendon not directly receiving muscular fibers was removed. The muscle weights from the "nonexercised" left limb were then compared to those from the exercised right limb of the trained animals using the t -test distribution. A probability of less than 0.05 was considered significant. The muscle weights of 5 control and 5 trained animals were also compared for the nonexercised control animals.

The histochemical examination was performed on the right and left FCR of 6 control and 3 trained animals. Fresh frozen serial sections (8 μ m) were processed for histochemically demonstrable alkali acid-stable myosin adenosine triphosphatase (ATPase) activity (Guth and Samaha 1970), reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH TR) activity (Novikoff *et al.* 1961), and iron for glycogen using the PAS staining technique. This muscle was chosen for detailed study because of its small size, and it has been demonstrated to undergo consistent hypertrophy (Gonyea and Ericson 1971). The total number of muscle fibers was counted from a photographic montage taken from a transverse section through comparable regions at the largest girth of the right and left FCR (Sola *et al.* 1971). In this way the total number of slow-twitch oxidative (SO) and fast-twitch fibers was decided. The distribution of fast-twitch fibers among subgroups, fast-twitch-oxidative glycolytic (FOG) or fast-twitch-glycolytic (FG) (Peter *et al.* 1972), was decided from 6 selected areas of the photo-montage during simultaneous microscopy of the original histochemical sections. The 6 selected areas contained a total of 500 fast-twitch fibers.

Results and Discussion

The exercise regimen used in this study has already been demonstrated to induce significant muscle hypertrophy (from 7 to 34%) in the exercised limb (Gonyea and Ericson 1971). In this study the control animals showed no significant difference in wet weight comparison between left and right FCR ($p > 0.05$). However in the trained cats, the weight of the exercised right FCR was significantly greater than that of the nonexercised left limb ($p < 0.05$).

The number of muscle fibers and the distribution among slow-twitch and fast-twitch fibers in FCR showed no significant difference among right and left sides in the control animals (Table 1). In the trained animals, however, there was a significant increase, 19%, in the total number of fibers of the exercised FCR when compared with that of the nonexercised left FCR ($p < 0.05$). This is a substantial difference when compared with the nonexercised cats (2.30%). It can also be observed that the average number of fibers in the right FCR is slightly greater in the exercised animals than that from the control animals even though the average weight of the control animals was greater than that of the exercised animals. Slow-twitch oxidative fibers were 37.1% in the left limb and 34.6%

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Fig. 1. Spaced serial sections of trained muscle (alcohol-stable ATPase) showing successive stages (a-f) in the splitting of fast-twitch-glycolytic fibers.

to the authors' knowledge, this is the first time fiber splitting has been demonstrated to occur during a training regimen not inducing degenerative changes of muscle fibers, and under a condition where the unrestrained animal performs weight-lifting exercise during voluntary contractions.

The present results also show definite muscle hypertrophy due to training. The lack of significant muscle hypertrophy during training of unrestrained animals without previous surgical intervention so often reported in the literature (*cf.* Peter 1971, Edgerton *et al.* 1972) is most likely due to the endurance type of training (treadmill running, swimming) used in these studies. As shown by Gonyea and Ericson (1976), and in the present study, weight-lifting exercise in the unrestrained animal induces significant muscle hypertrophy.

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As L. Wright and choline acetyltransferase activity of parotid glands in untreated rats and in rats treated with pilocarpine for 10 days. For the estimations of the enzyme activity the glands of each rat were pooled. Number of observations is indicated in brackets. Values are mean \pm S.E.

	Wet weights mg	Pilocarpine/Control percentage	Enzyme activity in μ g ACh/h/pooled glands	Pilocarpine/Control percentage	Enzyme activity in μ g ACh/h/g acronose powder	Pilocarpine/Control percentage
Control	133.8 \pm 3.7 (16)		39.3 \pm 1.9 (8)		740 \pm 30 (8)	
Treated	130.3 \pm 6.3 (16)	103.9 \pm 3.4 ^a	42.3 \pm 1.7 (8)	93.0 \pm 1.9 ^a	606 \pm 44 (8)	92.4 \pm 1.3 ^a

III 0.02, 0.01 less the glands of the pilocarpine treated rats compared with those of the treated control later state.

removed for 3 1/3-4 h and from the submandibular gland for 4-5 h in 2 rats examined. The values of salivary flow in one of the rats 0.59 ml and from the parotid gland 1.53 ml and from the submandibular gland, and in other rat 0.81 ml and 2.23 ml, respectively. Similar relationship between the two glands as to the amount of saliva secreted as response to an injection of pilocarpine was found by Holloway and Williams (1965).

contrast to larger doses of pilocarpine, the dose of 1 mg did not cause any diarrhoea in the animals. The rats were killed either, the drug treated rats 9-12 h after the last injection of pilocarpine. The parotid glands were then carefully taken out, cleaned, washed in saline, pressed gently between grease pads and weighed. For the estimation of choline acetyltransferase activity the parotid glands of each animal were weighed. The acronose dried powder of the glands as made up in citrate-saline in a concentration of 10% (Hobbs 1963, 1965). The activity of choline acetyltransferase was determined according to the method of Hobbs (1963, 1965). Of the tissue extract 0.2 ml was incubated for 1 h at 38°C. The acetylcholine formed was extracted on the frog rectum and expressed in μ g acetylcholine chloride formed per h per pool of glands (enzyme activity) and in μ g acetylcholine formed per h per g acronose powder (concentration); the tissue extracts from the drug treated rat and the untreated later state were incubated simultaneously and the fractions were assayed on the same rectum muscle.

The weights of the glands were expressed both in terms of wet weights and dry weights (acronose dried powder).

For statistical analysis Student's t-test was used, paired comparisons were made between the treated rats and its untreated later state. P values less than 0.05 were considered significant.

Results

Neither at the start nor at the end of the experimental period were there any differences in body weights between the drug treated rats and their untreated controls.

As shown in Table I the wet weights of the parotid glands were unchanged after the treatment with pilocarpine; this was also the finding when the dry weights were considered. It is also shown in the table that the total enzyme activity decreased ($p < 0.01$) in the parotids of the drug treated rats by about 7% and that the enzyme concentration decreased ($p < 0.02$) by about 8%.

Discussion

It appears reasonable to attribute the lowered choline acetyltransferase activity found in the parotid glands to decreased traffic of secretory impulses along the parasympathetic nerve of the glands; the activation of oral and pharyngeal receptors was probably reduced due to the secretion of saliva into the mouth evoked by the action of the parasympathomimetic

Choline Acetyltransferase Activity in Parotid Glands of Rats after Prolonged Treatment with Pilocarpine

By

JÖRGEN EKSTRÖM

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Abstract

Ekström, J. *Choline acetyltransferase activity in parotid glands of rats after prolonged treatment with pilocarpine* Acta physiol. scand. 1977 99 110-112.

The enzyme activity expressed as total activity and concentration, was found to be decreased in the cholinergic neurones of the glands after treatment with pilocarpine for 10 days. The secretion of saliva elicited by the parasympathomimetic drug is thought to have reduced the reflex stimulation of the glands by cholinergic secretory nerves and thereby lowered the enzyme activity in the nerves.

In parotid glands of the rat the activity of the acetylcholine synthesizing enzyme, choline acetyltransferase has been found to change when the consistency of the food is altered. The activity decreases when the animals are kept on a liquid diet (Ekström 1973), while it increases when the diet is dry and bulky (Ekström 1974 a). Further procedures causing dryness of the mouth such as treatment with an atropine-like drug or salivary duct ligation are followed by increased enzyme activity in these glands (Ekström 1974 a, 1975 a). The experiments support the concept that the activity of choline acetyltransferase is dependent on the traffic of impulses along a nervous pathway (see Ekström 1973 b 1976) when the reflex stimulation from the mouth is diminished the enzyme activity in the cholinergic neurones of the glands falls, while it rises when the stimulation is augmented.

The parasympathomimetic drug pilocarpine is known to cause a profound salivation and in the present study the effect of a prolonged treatment with this drug on the choline acetyltransferase activity in the parotid glands of the rat was examined.

Methods

Female rats, 7 months old and weighing about 200 g, of Sprague Dawley strain bred at this Institute were used. The rats were given a standard pelleted diet and water *ad libitum*. Pilocarpine hydrochloride, 1 mg/0.2 ml saline, was given s.c. every 6 h for 10 days to 8 rats. Controls served litter mates to the drug treated animals. In control experiments rats were anesthetized with chloralose and fine glass cannulae were inserted into the ducts of the parotid and the submaxillary glands. The dose of pilocarpine used as low as 0.1 mg/kg of body weight within 1 min from both types of glands. The secretion from the parotid gland

Influence of Acetate on the Metabolism of β Hydroxybutyrate in the Perfused Hind-Quarter of the Rat

By

N. KARLSSON, E. FELLENIUS and K.-H. KIRSELING

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Abstract

KARLSSON, N., E. FELLENIUS and K.-H. KIRSELING. *Influence of acetate on the metabolism of β -hydroxybutyrate in the perfused hind-quarter of the rat* Acta physiol. scand. 1977 99 113-122.

The effect of acetate, at concentrations normally found during ethanol combustion in intact animals and man, on the uptake and oxidation of β -hydroxybutyrate by the perfused rat hind-quarter was studied. The addition of acetate did not significantly affect the total uptake of β -hydroxybutyrate, but caused a 40% increase in the oxidation of β -hydroxybutyrate to CO_2 . The oxidation of ^{14}C - β -hydroxybutyrate to $^{14}\text{CO}_2$ accounted for about 10% of the total oxygen consumption by the perfused muscle in the absence of acetate. The presence of acetate this figure was reduced to about 5%. The addition of β -hydroxybutyrate did not significantly affect the metabolic flux of ^{14}C -acetate. It is concluded that acetate is preferred as oxidizable substrate to β -hydroxybutyrate. The combined oxidation of β -hydroxybutyrate by acetate did not affect the concentration ratio between β -hydroxybutyrate and acetoacrylate in the medium at the end of the perfusion, indicating that the ability of the muscle tissue to restore an increased redoxlevel, "exported" from the liver during ethanol combustion to extrahepatic tissues, was not impaired by acetate.

Key words: Acetate metabolism, ketone body metabolism, skeletal muscle, muscle perfusion, ethanol consumption.

The interaction between ethanol and ketone-body metabolism in intact animals and man is still an unsettled problem. In the liver the major site of ketone-body production (Williamson and Hems 1970), ethanol inhibits ketone-body production by diverting free fatty acids to triglyceride synthesis (Fellenius *et al.* 1973) and by direct inhibition of the β -oxidation step (Williamson *et al.* 1969, Fellenius and Kacaling 1973). In addition, ethanol combustion may strongly impair the oxidation of acetyl-CoA in the citric acid cycle (Majchrowicz 1975).

The combustion of ketone-bodies is known to be of significant importance for the energy-metabolism of the resting skeletal muscle (Ruderman *et al.* 1971, Williamson and Hems

Present address: Department of Analytical Chemistry and Biochemistry AB Hålsjö, S-431 20 Mölndal, Sweden.

Present address: Institute of Animal Nutrition, Agricultural College, S-750 07 Uppsala, Sweden.

drug. The decrease in the total enzyme activity by about 7% was less than that found in parotid glands of rats maintained on a liquid diet for 4–18 days, where the activity was reduced by 22–28% (Ekström 1973). The relatively small decrease observed might be explained by the fact that the pilocarpine treatment chosen was not sufficient to cause a continuous secretion throughout the 24 h, as judged from the results of the control experiments in which the salivary ducts were cannulated and the secretion of saliva studied after a single injection of pilocarpine. Larger doses or shorter intervals between the injections than those used were considered unsuitable not only because side effects such as diarrhoea would have appeared but also because a stimulating action of the drug on the autonomic ganglia could then be expected. Both the amylase-rich secretion and the weight increase of the parotid found after administration of big doses of pilocarpine to rats are known to be β -adrenergic effects mediated via the superior cervical ganglion (Schneyer 1965, 1969; Schneyer and El 1965). There was no glandular enlargement in the present study. Schneyer (1969) injected 10 mg per day (divided into 3 doses) for 111 days also and found an increase in weight of 22%. Obviously it is difficult to exclude the possibility of some action of the drug at the ganglionic level. If such an effect were to be exerted on the parasympathetic ganglia it could mean that nervous impulses were set up, thereby influencing the choline acetyltransferase activity and counteracting the effect of pilocarpine aimed at. When considering the effect on the sympathetic ganglion, it should be mentioned that stimulation of the β -adrenoceptors of the parotids with isoprenaline over a long time period is not followed by any changes in the total choline acetyltransferase activity (Ekström 1974 b).

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TABLE I. Rates of β -hydroxybutyrate, acetate and oxygen uptake and net production of lactate in the perfused hind-quarter of the rat.

Substrate added to medium	β -hydroxybutyrate uptake	Acetate uptake ($\mu\text{mol/h g wet weight}$)	Oxygen consumption	Net production of lactate
Control	—	—	27.3 ± 1.4 (5)	0.1 ± 0.8 (5)
Acetate, 2 mM	—	8.0 ± 0.5 (7)	30.6 ± 1.8 (6)	$2.3^* \pm 0.4$ (7)
β -hydroxybutyrate, 75 mM	0.87 ± 0.13 (8)	—	$25.5^{**} \pm 1.1$ (8)	$1.4^* \pm 0.3$ (8)
Acetate, 2 mM + β -hydroxybutyrate, 75 mM	0.79 ± 0.10 (10)	7.3 ± 0.2 (8)	$29.8^{**} \pm 0.7$ (12)	$2.1^* \pm 0.4$ (9)

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Rat hind-quarters were perfused for 70 min. The standard medium contained glucose, 5.5 mM. Substrates were added in 1) as a single dose (D.L. β -hydroxybutyrate) or as a single dose, followed by coenzyme infusion (acetate 0 $\mu\text{mol/h g wet weight}$ of perfused muscle) (Karlsson *et al.* 1975).

Data taken from Karlsson *et al.* (1976).

*Significantly different from perfusion with acetate alone (β -hydroxybutyrate, $p < 0.01$).

**Significantly different from perfusion with β -hydroxybutyrate alone (acetate, $p < 0.01$).

†Significantly different from control, $p < 0.05$.

muscle and treated with HClO_4 as before (Karlsson *et al.* 1975). The perchloric acid extract obtained was analysed for adenosine-triphosphate (ATP) (Lamprecht and Trustschfeld 1962), adenosine-diphosphate (ADP) and adenosine-mono-phosphate (AMP) (Adair 1962), creatine-phosphate (CP) (Lamprecht and Adair 1962), inorganic phosphate (P_i) (Bergmeyer *et al.* 1970) and lactate and pyruvate (Hohorst *et al.* 1979).

Radioactivity measurements

The metabolites from the added substrates were studied with ^3H -acetate and D.L. β -hydroxy(^3H)-pyruvate. The labelled substrates were added to give specific activity of 9 d.p.m./ μmol of substrate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson *et al.* 1976). The recoveries of added radioactivity are given in Table II and III. Uptake of label from the medium was calculated from the change in substrate concentration and the specific activity of the added labelled substrates.

Statistical analysis

Student's *t*-test was used for statistical analysis, $p < 0.05$ being considered statistically significant.

Results

Uptake of β -hydroxybutyrate, acetate and oxygen and the net production of lactate

The uptake of β -hydroxybutyrate from the medium (Table I) was linear during the experimental period. The net production of acetoacetate did not exceed 3% of the total uptake of β -hydroxybutyrate whether acetate was present or not (control value $0.04 \pm 0.04 \mu\text{mol/h g}$ (8)) in the presence of acetate $0.04 \pm 0.01 \mu\text{mol/h g}$ (10)). The addition of acetate did not change the uptake of β -hydroxybutyrate (Table I). Acetate was continuously infused into the perfusion medium to maintain a constant level of acetate during the experimental period, imitating the situation during administration of ethanol to man and animals *in vivo* (Majchrowicz 1975). The addition of β -hydroxybutyrate caused no significant changes in acetate uptake (Table I).

1970). It has also been shown previously that acetate, the major product of hepatic ether oxidation (Lundquist 1962) is readily oxidized by skeletal muscle (Karlsson *et al.* 1975). The administration of ethanol to animals and man *in vivo* is generally followed by a 15-fold elevation of the concentration of acetate in the peripheral blood (Majchrowski 1977). The extent to which acetate interferes with glucose and palmitate metabolism in the rat skeletal muscle has been outlined recently (Karlsson *et al.* 1976a, b). The present study was performed to investigate the interaction between acetate and β -hydroxybutyrate in the perfused hind-quarter of the rat.

Material and Methods

Animal treatment

Female Sprague Dawley rats, weighing 200–240 g, purchased from Anticimex, Sollefteå, Sweden, were used. The rats were fed *ad libitum* on a standard small animal diet (Astra Omnia, Södertälje, Sweden) and had free access to water prior to the experiments.

Reagents

Standard analytical grade laboratory reagents were obtained from E. Merck AG, Darmstadt, West Germany. Enzymes and co-enzymes were obtained from Biochimica Boehringer Mannheim, West Germany. Sigma Chemical Co., St. Louis, Miss., U.S.A. Solutions of hexobarbital (Eli Lilly and Co., Indianapolis, U.S.A.) (100 mg/ml) were freshly prepared from hexobarbital powder. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceuticals Co. Ltd, Eastbourne, England. Stock solutions of albumin (10%) were prepared in Krebs-Henseleit high bicarbonate buffer (Krebs and Henseleit 1932) and dialyzed at 4°C against 500 ml volume of the same buffer for 3–24 hr.

[U-¹⁴C]-acetate (59 mCi/mmol) and [3-¹⁴C]- β -hydroxybutyrate (4.7 mCi/mmol) were purchased from the Radiochemical Center, Amersham, England. Chemicals for measurement of radioactivity were obtained from Packard Instr. Co. Inc., Ill., U.S.A. and Koch Light Laboratories Ltd, Buckinghamshire, England.

Perfusion technique and experimental procedure

The method of rat hind-quarter perfusion used was that described by Ruderman *et al.* (1971). All perfusions were done on skinned animals with the hind legs at rest. The perfusion medium was the same as used previously (Karlsson *et al.* 1975). The final volume of the medium was 170 ml. The initial concentration of lactate in the medium was about 1.6 mM. Glucose consumption and lactate production due to glycolysis in the bovine red cells during the experimental period was negligible.

The rats were anaesthetized by intra-peritoneal injections of hexobarbital (25 mg/100 g b.wt.). The first 25 ml of the perfusate was discarded and the medium was then recycled. Substrates to be studied were added at 10 min. D,L- β -hydroxybutyrate (0.2 M stock solution, water) was added as a single dose to give a final concentration of 0.75 mM of the D-isomer in the medium. Acetate (1.5 M stock solution in water) was added as single doses, followed by continuous infusion of acetate in the medium at a rate of 7.0 μ mol/h (Karlsson *et al.* 1975). Samples of the medium for analysis of acetate, acetoacetate, β -hydroxybutyrate, lactate and pyruvate were taken at 15, 40 and 70 min.

Analysal methods

Samples of the medium for analysis of the concentrations of acetoacetate, β -hydroxybutyrate, lactate and pyruvate were deproteinized in ice-cold 0.6 M HClO₄. The extracts were neutralized with KOH and the precipitated KClO₄ was removed by centrifugation. The concentrations of acetoacetate (Mellanby and Williamson 1970), β -hydroxybutyrate (Williamson and Mellanby 1970) and lactate and pyruvate (Hohorst *et al.* 1959) were measured in these extracts. Acetate was determined in plasma according to the method of Bergmeyer and Möllering (1970). A freshly prepared standard solution of acetate (2 mM) in albumin (4%) was always run parallel to the plasma samples, when acetate was determined. Determination of oxygen consumption by the perfused tissue was performed with polarographic technique as described previously (Karlsson *et al.* 1975). Oxygen measurements are usually done at 25, 35, 50 and 60 min.

At the end of the perfusion a portion of the thigh muscles was rapidly frozen *in situ* with aluminium tongs cooled in liquid nitrogen (Wollenberger *et al.* 1960). The frozen tissue (300 mg) was

TABLE 1. Rates of β -hydroxybutyrate, acetate and oxygen uptake and net production of lactate in the perfused hind-quarter of the rat

Infusions to perfused medium	β -hydroxybutyrate uptake	Acetate uptake (μ mol/h g wet weight)	Oxygen consumption	Net production of lactate
control	—	—	27.3 ± 1.4 (5)	0.1 ± 0.3 (5)
control, 20 mM inulin	—	8.0 ± 0.5 (7)	30.6 ± 1.8 (6)	$2.3^a \pm 0.4$ (7)
hydroxybutyrate 75 mM	0.87 ± 0.13 (8)	—	$25.3^a \pm 1.1$ (8)	$1.6^a \pm 0.3$ (8)
acetate, 20 mM inulin	0.79 ± 0.10 (10)	7.3 ± 0.2 (6)	$29.8^a \pm 0.7$ (12)	$2.1^a \pm 0.4$ (9)
hydroxybutyrate 75 mM	—	—	—	—

Values are expressed as mean \pm S.E. with the number of observations in parentheses. Rat hind-quarters were perfused for 70 min. The standard medium contained glucose, 5.5 mM. Substrates were added as mix or single doses (D.L. 3-hydroxybutyrate) or as single doses, followed by continuous infusion (Acetate) (μ mol/h g wet weight of perfused muscle) (Karlsson *et al.* 1975).

Data taken from Karlsson *et al.* (1976).

^a Significantly different from perfusion with acetate alone (β -hydroxybutyrate, $P < 0.01$).

^b Significantly different from perfusion with β -hydroxybutyrate alone (acetate, $P < 0.01$).

^c Significantly different from control, $P < 0.05$.

was added and treated with HClO₄ as before (Karlsson *et al.* 1975). The perchloric acid extract obtained was analysed for adenosine-triphosphate (ATP) (Lamprecht and Trautwein 1962), adenosine-diphosphate (ADP) and adenosine-monophosphate (AMP) (Adem 1962), creatine-phosphate (CP) (Lamprecht and Trautwein 1962), inorganic phosphate (P_i) (Baginski *et al.* 1970) and lactate and pyruvate (Hohorst *et al.* 1973).

Isotopically determined

The metabolic fates of the added substrates were studied with U-¹⁴C-acetate and D.L. 3-hydroxy(3-¹⁴C)-butyrate. The labelled substrates were added to give specific activity of 9 μ Ci/ μ mol of substrate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson *et al.* 1975, Karlsson *et al.* 1976). The recoveries of added radioactivity are given in Table II and III. Uptake of label from the medium was calculated from the change in substrate concentration and the specific activity of the added labelled substrates.

Statistical analysis

Student's *t*-test was used for statistical analysis, $P < 0.05$ being considered statistically significant.

Results

Uptake of β -hydroxybutyrate, acetate and oxygen and the net production of lactate

The uptake of β -hydroxybutyrate from the medium (Table I) was linear during the experimental period. The net production of acetate did not exceed 5% of the total uptake of β -hydroxybutyrate whether acetate was present or not (control value 0.04 ± 0.04 μ mol/h g (3)) or in the presence of acetate (0.04 ± 0.01 μ mol/h g (10)). The addition of acetate did not change the uptake of β -hydroxybutyrate (Table I). Acetate was continuously infused into the perfusion medium to maintain a constant level of acetate during the experimental period, imitating the situation during administration of ethanol to man and animals *in vivo* (Majchrowicz 1975). The addition of β -hydroxybutyrate caused no significant changes in its uptake (Table I).

TABLE II The effect of acetate on the metabolic fate of D,L-3-hydroxy(3-¹⁴C)butyrate in the perfused hind-quarter of the rat

Additions to standard medium	β -hydroxybutyrate uptake (μ mol/h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of ¹⁴ C- β -hydroxybutyrate to ¹⁴ CO ₂ (μ l ¹)	Recovery added label (%)
		¹⁴ CO ₂ (μ mol of β -hydroxybutyrate/h g wet weight)	Lipids (μ mol of β -hydroxybutyrate/h g wet weight)		
¹⁴ C β -hydroxybutyrate 0.75 mM	0.97 \pm 0.16 (6)	0.45 \pm 0.03 (6)	0.033 \pm 0.004 (6)	8.1 \pm 0.4 (6)	91 \pm 1
¹⁴ C β -hydroxybutyrate 0.75 mM	0.80 \pm 0.13 (6)	0.77 \pm 0.02 (6)	0.029 \pm 0.008 (6)	4.1 \pm 0.4 (6)	94 \pm 1
Acetate, 2 mM + infusion					

Results are expressed as mean \pm S.E. with the number of observations in parentheses.

Experimental conditions see Table I and Methods.

¹ Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

Significantly different from perfusion without acetate, $p < 0.01$.

Acetate alone did not significantly affect the oxygen consumption by the perfused tissue (Table I). β -hydroxybutyrate lowered the oxygen consumption, as compared to the situation with acetate and this lowering was restored to the control value by the addition of acetate.

In the control experiments, when no substrate was added to the standard medium, the lactate concentration showed only small changes during the perfusion. When acetate, β -hydroxybutyrate or both were added to the medium there was a net production of lactate by the perfused tissue (Table I).

Influence of acetate on ¹⁴C β -hydroxybutyrate metabolism

The β -hydroxybutyrate taken up from the medium was readily oxidized in the perfused muscle. Thus, about 50% of the ¹⁴C label taken up was found in ¹⁴CO₂, while only small amounts were found in lipids in the chloroform extract of the muscle tissue (Table II). About 40% of the total uptake of β -hydroxybutyrate was found as water-soluble metabolite in the PCA-extract of the muscle tissue. When acetate was added the oxidation of ¹⁴C β -hydroxybutyrate was significantly decreased (Table II). However, in relation to the total uptake of β -hydroxybutyrate from the medium, there was no statistically significant effect of acetate on the oxidation of the labelled substrate to ¹⁴CO₂. This was probably due to the tendency towards a slightly decreased uptake of β -hydroxybutyrate in the presence of acetate in these experiments. The incorporation of ¹⁴C-label into lipids was not changed by acetate. Neither was there any change in radioactivity in the PCA-extract of the muscle tissue.

The complete oxidation of 1 mol of β -hydroxybutyrate requires 4.5 mol of O₂. Therefore, with the known rates of oxidation of β -hydroxybutyrate (Table II) and the total oxygen consumption (Table I) it can be calculated that the combustion of ¹⁴C β -hydroxybutyrate to ¹⁴CO₂ accounted for about 8% of the total oxygen consumption by the muscle tissue (Table II). In the presence of acetate this figure was decreased to 4%.

TABLE II. The effect of acetate on the metabolic fate of D.L. 3-hydroxy(3-¹⁴C)butyrate in the perfused hind-quarter of the rat.

Additions to standard medium	β hydroxybutyrate uptake (μ mol/h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of ¹⁴ C β -hydroxybutyrate to ¹⁴ CO ₂ (%) ¹	Recovery of added label ()
		¹⁴ CO ₂ (μ mol of β -hydroxybutyrate/h g wet weight)	Lipids (μ mol of β -hydroxybutyrate/h g wet weight)		
¹⁴ C β hydroxybutyrate 0.75 mM	0.97 \pm 0.16 (6)	0.45 \pm 0.03 (6)	0.033 \pm 0.004 (6)	8.1 \pm 0.4 (6)	98 \pm 2
¹⁴ C β -hydroxybutyrate 0.75 mM Acetate mM + infusion	0.80 \pm 0.13 (6)	0.27 ^a \pm 0.02 (6)	0.029 \pm 0.006 (6)	4.1 ^a \pm 0.4 (6)	94 \pm 1

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions, see Table I and Methods.

¹ Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

^a Significantly different from perfusion without acetate, $p < 0.01$

Acetate alone did not significantly affect the oxygen consumption by the perfused tissue (Table I). β -hydroxybutyrate lowered the oxygen consumption as compared to the situation with acetate and this lowering was restored to the control value by the addition of acetate.

In the control experiments, when no substrate was added to the standard medium, the lactate concentration showed only small changes during the perfusion. When acetate or β -hydroxybutyrate or both were added to the medium there was a net production of lactate by the perfused tissue (Table I).

Influence of acetate on ¹⁴C β -hydroxybutyrate metabolism

The β -hydroxybutyrate taken up from the medium was readily oxidized in the perfused muscle. Thus, about 50% of the ¹⁴C-label taken up was found in ¹⁴CO₂, while only small amounts were found in lipids in the chloroform extract of the muscle tissue (Table III). About 40% of the total uptake of β -hydroxybutyrate was found as water-soluble metabolites in the PCA-extract of the muscle tissue. When acetate was added the oxidation of ¹⁴C- β -hydroxybutyrate was significantly decreased (Table II). However, in relation to the total uptake of β -hydroxybutyrate from the medium there was no statistically significant effect of acetate on the oxidation of the labelled substrate to ¹⁴CO₂. This was probably due to a tendency towards a slightly decreased uptake of β -hydroxybutyrate in the presence of acetate in these experiments. The incorporation of ¹⁴C label into lipids was not changed by acetate. Neither was there any change in radioactivity in the PCA-extract of the muscle tissue.

The complete oxidation of 1 mol of β -hydroxybutyrate requires 4.5 mol of O₂. Therefore, with the known rates of oxidation of β -hydroxybutyrate (Table II) and the total oxygen consumption (Table I) it can be calculated that the combustion of ¹⁴C β -hydroxybutyrate to ¹⁴CO₂ accounted for about 8% of the total oxygen consumption by the muscle tissue (Table II). In the presence of acetate this figure was decreased to 4%.

No.	PYRUVATE	L/P	β -OH-bu/acet
0.14 (5)	0.11 ± 0.02 (5)	20.7 ± 4.4 (5)	—
0.13 (6)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)	—
0.23 (8)	0.14 ± 0.01 (8)	16.1 ± 2.1 (8)	24.8 ± 6.9 (6)
0.13 (9)	0.12 ± 0.01 (9)	19.2 ± 1.7 (9)	19.5 ± 3.5 (10)

P	$\frac{ATP}{ADP \cdot P}$	$\frac{ADP^i}{ATP \cdot AMP}$
1.5 (4)	16.0 ± 1.2 (6)	0.46 ± 0.03 (6)
1.6 (5)	15.1 ± 1.4 (6)	0.51 ± 0.07 (4)
± 1.2 (7)	$12.8^a \pm 0.3$ (7)	$0.60^a \pm 0.02$ (7)
4.6 (8)	$14.7^b \pm 0.7$ (8)	0.54 ± 0.04 (8)

The ratio $[ADP^i]/[ATP] \cdot [AMP]$, which represents the mass action ratio of the adenylate kinase reaction, was not significantly altered by the presence of β -hydroxybutyrate or acetate or both (Table V).

Discussion

It was found in the present investigation that the total uptake of β -hydroxybutyrate by the perfused rat hind-quarter (Table I) was slightly higher than that found by Ruderman *et al.* (1971) and Ruderman and Goodman (1973) with the same experimental technique. We found, using radioactively labelled β -hydroxybutyrate, that about 45% of the substrate taken up from the perfusion medium was oxidized to CO_2 and $3H_2O$ (Table II). Similar results have been found by Balasse and Havel (1971) for acetoacetate oxidation in peripheral tissues. In addition, Hagenfeldt and Walgren (1968) found that about 25% of β -hydroxybutyrate taken up by the human forearm was oxidized to CO_2 . Furthermore, the contribution

TABLE IV The effect of acetate or β -hydroxybutyrate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused rat hind-quarter at the end of the perfusion. The [β -hydroxybutyrate]/[lactate+acetate] (β -OH but/AcAc) ratio in the medium at the end of the perfusion is also shown.

Additions to standard medium	Muscle tissue		
	Lactate (μ mol/g wet weight)	Pyruvate	L/P
Control ¹	1.15 \pm 0.20 (6)	0.14 \pm 0.02 (6)	7.6 \pm 1.4
Acetate, 2mM ¹ +infusion	2.21 \pm 0.47 (4)	0.08 ^a \pm 0.01 (6)	27.4 ^a \pm 9.4
β -hydroxybutyrate 0.75 mM	1.73 ^a \pm 0.15 (7)	0.09 ^a \pm 0.01 (7)	20.3 ^a \pm 3.1
β -hydroxybutyrate 0.75 mM			
Acetate 2mM+infusion	1.30 ^a \pm 0.26 (8)	0.09 ^a \pm 0.01 (8)	30.2 ^a \pm 5.1

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

¹ Data taken from Karlsson *et al.* (1976 a).

^a Significantly different from control, $p < 0.05$.

^b As for a, $p < 0.01$.

Significantly different from perfusion with β -hydroxybutyrate without acetate, $p < 0.05$.

TABLE V The effect of acetate or β -hydroxybutyrate or both on the concentrations of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy-charge" in the muscle tissue of the perfused hind-quarter of the rat at the end of the perfusion.

Additions to standard medium	ATP			ADP			AMP (μ mol/g wet weight)		
Control ¹	5.01 \pm 0.31 (6)			0.70 \pm 0.05 (6)			0.04 \pm 0.004 (6)		
Acetate, 2mM ¹ +infusion	5.33 \pm 0.16 (6)			0.77 \pm 0.09 (6)			0.036 \pm 0.004 (6)		
β -hydroxybutyrate 0.75 mM	4.87 \pm 0.21 (7)			0.64 \pm 0.01 (7)			0.034 \pm 0.003 (7)		
β -hydroxybutyrate 0.75 mM	5.06 \pm 0.19 (8)			0.65 \pm 0.01 (8)			0.037 \pm 0.004 (8)		
Acetate, 2mM+infusion									

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

¹ Data taken from Karlsson *et al.* (1976 b).

Significantly different from control, $p < 0.05$.

^a Significantly different from perfusion with β -hydroxybutyrate without acetate, $p < 0.05$.

As for a, $p < 0.01$.

Tissue concentrations of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy-charge" in the muscle tissue

The tissue content of high-energy phosphate compounds (ATP, ADP and CP) was not changed by the addition of acetate or β -hydroxybutyrate or both to the medium, as compared to the control value (Table V). However, in the presence of acetate and β -hydroxybutyrate there was a statistically significant increase in AMP as compared to the control value (Table V). In addition, in the presence of β -hydroxybutyrate the tissue content of P_i was decreased, as compared to the control value. This effect caused an increase in the "phosphate potential" ([ATP]/[ADP] \cdot [P_i]) (Atkinson 1969). The increase in the "phosphate potential" caused by β -hydroxybutyrate, was restored to the control value by the addition of acetate.

and inorganic phosphate do not give any clear-cut answer how these compounds are involved in the metabolic interaction between β -hydroxybutyrate and acetate (Table V). During ethanol oxidation in intact animals the redox-state, measured as the concentration ratio between lactate and pyruvate or between β -hydroxybutyrate and acetoacetate, is increased in the liver tissue (Forsander 1970). This change is followed by alterations in several metabolic steps in the liver. The changes in the hepatic redox-state are generally reflected by similar changes in the hepatic venous blood and the increased redox-level is "exported" to extrahepatic tissues, carried in the blood stream (Forsander 1970, Fellenius et al. 1973 b). The present results demonstrate that, in spite of an inhibited oxidation of β -hydroxybutyrate, acetate did not impair the readjustment of the ratio [β -hydroxybutyrate]/[acetoacetate].

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of β -hydroxybutyrate oxidation to the oxidative metabolism in the perfused muscle is only about 8% (Table II). The low utilization of this substrate was probably due to a low activity of β -hydroxybutyrate dehydrogenase in skeletal muscle (Williamson and Hor 1970). The low activity of this enzyme is demonstrated by our finding of a slow establishment of an equilibration in the medium of the concentration ratio between β -hydroxybutyrate and acetoacetate. The ratio of 20-25 found at the end of the perfusion (Table II) should be compared with a ratio of 1-2 in rat blood at equilibration (Williamson and Hor 1970). Our finding of a relatively low contribution of β -hydroxybutyrate oxidation to the total oxidative metabolism in the perfused rat hindquarter at rest (Table II) is supported by those of Ruderman *et al* (1971) with the same experimental technique of Domini *et al* (1974) in sheep hindlimb and of Barton (1973) in the intact rat *in vivo*.

The addition of acetate to the perfusion medium caused a significant decrease in β -hydroxybutyrate oxidation (Table II). Since there was a tendency towards a decreased rate of uptake of β -hydroxybutyrate by acetate there was no statistically significant effect of acetate on the oxidation of β -hydroxybutyrate if the oxidation of β -hydroxybutyrate is related to the total uptake of β -hydroxybutyrate from the medium (Table II). However, the oxygen consumption due to oxidation of β -hydroxybutyrate, was significantly decreased by acetate. Thus, as may be seen from Table II, acetate decreased the contribution of β -hydroxybutyrate oxidation to the energy metabolism of the muscle tissue. This suggests that in our experiments β -hydroxybutyrate oxidation was impaired by the addition of acetate. Ruderman *et al* (1971), Goodman and Ruderman (1972) and Ruderman and Goodman (1973) found that the addition of octanoate or oleate to the perfusion medium did not affect the uptake of acetoacetate in the perfused rat hindquarter. However, it is not clear from these reports whether there were any effects of the added fatty acids on the oxidation of acetoacetate. It should be pointed out that at comparable concentrations acetoacetate is utilized more rapidly than β -hydroxybutyrate (Ruderman *et al* 1971, Ruderman and Goodman 1973). The pattern of interaction between acetoacetate and acetate might therefore, be different due to the high rate of utilization of acetoacetate, as compared to β -hydroxybutyrate. In accordance with our findings with β -hydroxybutyrate in rat skeletal muscle Lefèvre *et al* (1970) found no effect of acetate on the uptake of acetoacetate in the rat diaphragm.

Although there was a slight decrease in acetate uptake by β -hydroxybutyrate, we did not find any effect of β -hydroxybutyrate on the oxidation of acetate or the oxygen consumption due to oxidation of acetate to CO₂ (Table III). This suggests that acetate is preferred as oxidative substrate to β -hydroxybutyrate at the substrate concentrations used in the present study. Little *et al* (1970) found that infusion of β -hydroxybutyrate in dogs *in vivo* caused a decrease in the uptake of free fatty acids in the resting skeletal muscle and the myocardium. Olson (1962) and Bassenge *et al* (1965) found that acetoacetate had a similar effect in the perfused rat heart. The differences between the results obtained in this study and those cited above, concerning the interaction between ketone bodies and free fatty acids, might well be explained by differences in the metabolism of the tissues studied, the ketone body used (β -hydroxybutyrate or acetoacetate) and the concentration of the ketone bodies and the short- or long-chain free fatty acid.

The results of analysis of the redox substrates, adenine-nucleotides, creatine phosphate

Training Induced Changes in the Subgroups of Human Type II Skeletal Muscle Fibres

By

PER ANDERSEN and JAN HENRIKSSON

Intense endurance training causes no change in muscle fibre type distribution in neither animals nor humans, when fibres are classified into 2 main groups according to contractile properties (Bernard *et al.* 1970, Gollnick *et al.* 1973). On the other hand, an increase in the percentage of the high oxidative at the expense of the low oxidative fast twitch fibres has been reported to occur in animals (Bernard *et al.* 1970). In endurance trained humans, the fast twitch (type II) muscle fibre population has been found almost exclusively to consist of high oxidative (type IIA) fibres (Jansson 1973, Nygaard Jensen 1976). Whether this predominance of type IIA fibres is hereditary or induced by training is not known.

The aim of the present study was to analyze muscle fibre type distribution, with special reference to the subgroups of type II fibres, before and during a period of intense endurance training.

Material and Methods

11 healthy volunteers (age 20-25 years, weight 57-110 kg and height 1.60-1.90 m) participated in the study. They were all fully informed of the risks and discomfort associated with the experiments before they volunteered to participate. All had sedentary occupations and none had been engaged in any regular physical training during the preceding year.

The subjects trained for 8 weeks by pedalling bicycle ergometer on average 30 min day 4 times week. The average work intensity corresponded to 81 % $\dot{V}O_{2\max}$ (mean \pm S.E., range 71-88 %). Before and after the training period maximal oxygen uptake ($\dot{V}O_{2\max}$) was determined, with the subjects pedalling bicycle ergometer and muscle biopsies (20-40 mg each) were taken from the quadriceps femoris muscle (vastus lateralis) using needle biopsy technique (Bergström 1962). On 3 of the subjects this procedure was also followed after 1, 2, 3, and 5 weeks of training.

On testing days the subjects always came to the laboratory in the morning, after light breakfast. All work and sleeping were performed about 72 h after the last training session.

A detailed description of the maximal oxygen uptake determinations is given elsewhere (Henriksson and Rasmussen 1976). The muscle samples were mounted in embedding medium (Araldex OCT), frozen in isopentane cooled with liquid nitrogen, and stored at -80°C before analysis. Serial transverse sections (10 μ m) were cut in microtome at -20°C, and stained histochemically for myofibrillar adenosine triphosphatase (ATPase) by incubation in glycine buffer at pH 9.4, after acid or alkaline preincubation in either acetate or glycine buffer. To classify fibres into *myo*-actin groups, type I and type II, and to identify type IIC fibres (Debold and Brooke 1973), preincubation at pH 4.4 (5 min, 21°C) and at pH 10.3 (8 min, 37°C) was used (Brooke and Kaiser 1968). For classification into subgroups IIA and IIB (Brooke and Kaiser 1970) post incubation at both pH 4.6 (30 min, 21°C) and pH 4.8 (5 min, 21°C) were used.

In most sections, small number of type II fibres was stained in an intermediate fashion, and could not with certainty be categorized as either type IIA or IIB. On the average these fibres made up 7% (range 0-21) of the total number of type II fibres, with no systematic variation during the course of the training period. For further treatment of the data half of these fibres in each section were considered as type IIA and half as type IIB.

The significance of interindividual differences was tested using the paired Student's *t*-test.

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percentage distribution of type IIA and IIB fibres was in the same range as that reported by Edberg and Jansson (1976) for 16 sixteen year-old boys (32% IIA and 13% IIB fibres).

It is worth emphasizing that the classification of the type II fibres into the subgroups IIA and IIB in this study is based on differences in the pH sensitivity of the myosin ATPase. According to Brooke and Kaiser (1970) this difference is related to the reactivity of sulfhydryl groups in the myosin molecule. Thus it is conceivable that the observed changes indeed indicate a change in the structure of the myosin molecule.

To give rise to conversion of type IIB to IIA fibres it is likely that, during the training, type IIB fibres are activated much more frequently than in the pre-training situation. Activation of type IIB fibres at exercise intensities comparable to those used in our training programme are in agreement with the results of Andersen and Sjøgaard (1976).

This study was supported by the Danish National Science Research Council.

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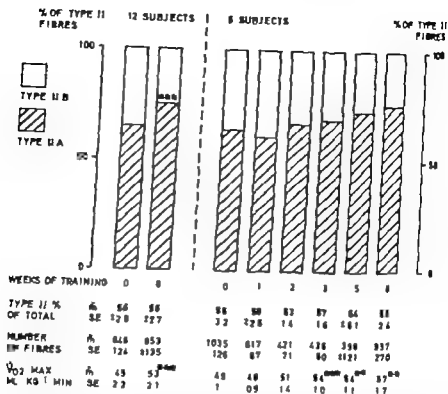


Fig. 1 Change in type II subgroups as percentage of the total number of type II fibres (type IIC fibres not included). In the lower part of the figure, per cent distribution of type II fibres, number of fibres counted, as well as maximal oxygen uptake relative to body weight is given. Significant differences from pre-training values are indicated ($p < 0.05$ $p < 0.01$ $p < 0.001$).

Results

Maximal oxygen uptake (\dot{V}_{O_2} max) The initial mean \dot{V}_{O_2} max (12 subjects) was 45 ml $\text{kg}^{-1} \times \text{min}$ (b wt. 74.4 kg) and increased by 18% to an average of 53 ml $\text{kg}^{-1} \times \text{min}$ (b wt. 74.6 kg) as a result of the training ($p < 0.001$). Individual improvement varied from 8% to 37%. \dot{V}_{O_2} max during the course of the training period (5 subjects) is given in Fig. 1. Also for these subjects body weight was unchanged with training (67.3 kg before vs 67.9 kg after).

Fibre type distribution For the whole group fibre distribution was 41% type I 37% type IIA, and 19% type IIB before as compared to 43% type I 42% type IIA and 14% type IIB after the training period. The observed differences in both type IIA and IIB percentages were statistically significant ($p < 0.05$). In order to focus on the type II population the number of type IIA fibres was also calculated in per cent of the total number of type II fibres. Expressed in this way the values are 65% before vs 75% after training ($p < 0.001$). The time course of the changes, for 5 subjects, is given in Fig. 1. The percentage of type IIC fibres did not change significantly with training (2.6% before vs 1.2% after training).

Discussion

The present results indicate a gradual conversion of a part of the type IIB fibre population into type IIA fibres in response to an endurance training programme. The pre-training per

percentage distribution of type IIA and IIB fibres was in the same range as that reported by edberg and Jansson (1976) for 68 sixteen year-old boys (32% IIA and 13% IIB fibres).

It is worth emphasizing that the classification of the type II fibres into the subgroups IIA and IIB in this study is based on differences in the pH sensitivity of the myosin ATPase. According to Brooks and Kaiser (1970) this difference is related to the reactivity of sulfhydryl groups of the myosin molecule. Thus it is conceivable that the observed changes indeed indicate a change in the structure of the myosin molecule.

To give rise to a conversion of type IIB to IIA fibres it is likely that, during the training, the type IIB fibres are activated much more frequently than in the pre-training situation. Activation of type IIB fibres at exercise intensities comparable to those used in our training regime are in agreement with the results of Andersen and Sjogaard (1976).

This study was supported by the Danish Natural Science Research Council.

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Vagal Release of Somatostatin into the Antral Lumen of Cats

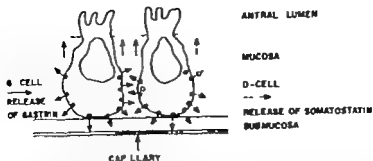
By

KERSTIN UVNÄS-WALLÉN, SUAD EFENDIC and ROLF LUFT

Large amounts of gastrin 17 have been demonstrated in the gastric juice of cats (Uvnäs-Wallén 1976, Uvnäs-Wallén and Rehfeld 1976). Andersson and Nilsson (1974) reported that gastrin is present in antral perfusates of dogs. In the cat gastrin is released directly into the antral lumen in parallel with the release of gastrin into the gastric venous outflow. Thus electrical vagal stimulation causes a release of gastrin both into the antral lumen and into the gastric venous blood (Uvnäs-Wallén to be published).

The occurrence of gastrin in the gastric lumen, as well as in the circulation, might be explained as due to gastrin being released diffusely into the interstitial fluid from the G-cells (Fig. 1). Gastrin might then exert local or paracrine effects on neighbouring cells, as well as blood borne remote endocrine effects. Somatostatin containing cells are located close to the G-cells in the antral mucosa and both types of cells have similar morphological characteristics. Therefore somatostatin might also be expected to be released into the antral lumen.

The only well established mechanism by which the release of gastrin can be inhibited is by low intra-antral pH. It has been assumed that the effect of low pH is exerted directly on the G-cells. The presence of somatostatin cells close to the G-cells in the antrum, and its ability to antagonize the release of gastrin (Bloom *et al.* 1974, Le Roith *et al.* 1975, Raptis *et al.* 1975, Uvnäs-Wallén *et al.* in press), suggested to us that the inhibitory effect of low pH might be mediated via a release of somatostatin. To test this hypothesis, the antrum of 5 cats was perfused with slightly alkaline or/and acid solutions and vagal stimulation (5 Hz) were performed during the perfusions. Perfusates were assayed for both somatostatin and gastrin immunoreactivity. Immediately upon collection, the pH of the sample was adjusted to 7, they were boiled for 15 min and then frozen until the hormone content was determined by radioimmunoassay. Since the antrum was perfused at a constant rate (1 ml/min) the output of hormone per minute could be calculated. In all experiments, vagal stimulation caused an increased release (100%) of somatostatin into the antral lumen during perfusion with 0.1 M HCl, when the output of gastrin was reduced to about 30%. During perfusion with a slightly alkaline medium, vagal stimulations almost failed to cause a release of somatostatin (7%), but large amounts of gastrin were released instead (100%) (Table 1). A typical experiment is illustrated in Fig. 2. On the average 351 ng of gastrin were released by vagal stimulation during perfusion with phosphate buffer (pH 7.8) while the maximal



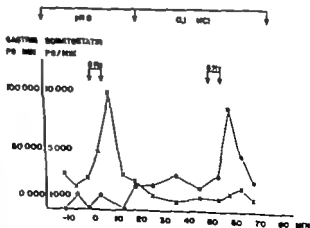
1 Schematic picture of gastrin containing cell (G-cell) and somatostatin containing cell (D-cell). Arrows illustrate how the two hormones might be released diffusely from the cells. This model could see how the hormones from one type of hormone producing cells can, at the same time, exert permissive endocrine actions and also appear in the gastrointestinal lumen.

ase of somatostatin, obtained during perfusion with 0.1 M HCl, was only about 7% (ng/bile l).

both gastrin and somatostatin can be released by vagal stimulation. The results with a procal occurrence of gastrin and somatostatin in the antral lumen suggest that the inhibitory effect exerted by HCl on the release of gastrin, might at least partially be due to local effect of somatostatin on the G-cells.

The antral gastrin and somatostatin producing cells probably release their hormones into the surrounding interstitial tissue, from which the hormones diffuse in all directions. Gastrin has been demonstrated to reach the antral lumen as well as the blood. Until now in response to vagal stimulation, somatostatin has only been demonstrated to occur in the antral mucosa. However, simultaneous release of the hormones might be expected to occur into the circulation.

The gastrointestinal mucosa contains a large number of different hormone producing cells. It might therefore be expected, that the hormones produced by these cells also will pass



2 Experiments in which the antrum was perfused with phosphate buffer pH 8 and 0.1 M HCl. Vagal stimulation (5 Hz for 5 min) was performed during perfusion with both solutions.

TABLE 1 Release of gastrin (G) and somatostatin (S) into the antral lumen during perfusion with 0.1 M HCl and/or phosphate buffer pH 7-8. The output of hormone was determined gravimetrically and expressed in ng. In experiments in which stimulations were performed at both pHs, the low output was expressed in % of the maximal output (100%). Note that the maximal output of gastrin (pH 7-8) was on the average 5 times higher than that of somatostatin (pH 1.2).

Exp. no.	pH 1		pH 7-8	
	g	%	ng	
1 G	75	14	525	100
S	46	100	0	0
2 G	118	37	320	100
S	6	100	3	1
3 G	60	38	160	100
S	79	100	3	10
4 G			400	
S			11	
5 G	34			
S	10			
Mean				
G	72	30	351	100
S	28	100		7

into the gastrointestinal lumen. Since many gastrointestinal hormones cannot be measured in the blood, due to methodological difficulties or to too low circulating hormone levels it might even be advantageous to follow the output of these hormones in gastrointestinal perfusates.

This study was supported by grants from Amundsons fond and Nordisk insamlings fond.

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Concave-like Membrane Structures and Olfactory Transduction

By

G. O. SPERBER

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Abstract

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Current theories concerning the olfactory transduction process are discussed. A hypothesis is formulated, according to which the olfactory receptor membrane contains regions, here it is the structure of lipid-protein concave-like structures, which occur in living cells. Such membrane could have the ability to change its permeability in response to odorants and sensitivity comparable to that of the sense of smell. The model also explains the fact that different receptor cells have different sensitivity patterns towards odorants. The model is consistent with the results of experiments that seek to establish the locus of olfactory action.

At present, it is usually accepted that the olfactory receptor cells function essentially as described by Ottoson (1970). According to this view the primary event in sniffing is some process whereby odorant molecules, entering the plasma membrane of the cilia or comparable structures of the receptor cell, cause some change in the ionic permeability of this membrane. The nature of this transduction process is unknown. In this paper the model for the transduction process put forward by Bungenberg de Jong and Saubert (1937) is reformulated and reevaluated in the light of new experimental data (Sperber in press) and present-day knowledge of the physiology of the sense of smell.

The many "theories of smell" do not concern themselves much with how the transduction process actually works. Most theories bypass the question entirely and some make a general assumption (such as membrane-active substances or "allosteric proteins") about it, which is not an integral part of the theory. In recent times, only two specific hypotheses about the olfactory transduction mechanism have been developed further than mere suggestions.

Rosenberg *et al.* (1968) suggested that the conductance of carotenoids in the membrane could be changed by the odorant molecules. They showed that the conductance of carotenoid crystals is actually very sensitive to many vapours. Against this attractively clear and

TABLE I Release of gastrin (G) and somatostatin (S) into the antral lumen during perfusion with 0.1 M HCl and/or phosphate buffer pH 7-8. The output of hormone was determined quantitatively and expressed in ng. In experiments in which stimulations were performed at both pHs, the output was expressed in % of the maximal output (100%). Note that the maximal output of gastrin (pH 7-8) was on the average 5 times higher than that of somatostatin (pH 1-2).

Exp. no	pH 1-2		pH 7-8	
	ng		ng	
1 G	73	14	525	100
S	46	100	0	0
2 G	118	37	320	100
S	26	100	3	12
3 G	60	38	160	100
S	79	100	3	10
4 G			400	
S			0	
5 G	34			
S	10			
Mean				
G	72	30	351	100
S	28	100	2	7

into the gastrointestinal lumen. Since many gastrointestinal hormones cannot be measured in the blood due to methodological difficulties or to too low circulating hormone levels it might even be advantageous to follow the output of these hormones in gastrointestinal perfusates.

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simple theory. It may be objected that there is some doubt whether any carotenoids are present in the receptor cells (Takagi and Yajima 1965), and that the conductance of the ionenoids so far investigated is too low.

The hypothesis of Davies and Taylor (1957) the "penetration and puncturing" theory assumes that when odorant molecules adsorbed into the lipid membrane desorb again, to leave a hole in the membrane for some time, through which ions may pass. This theory can predict odour quality to some extent (Davies 1970), but no model membrane has been for which actually shows such properties. Experiments on artificial bilayers have been somewhat disappointing (Cherry *et al.* 1970).

The contribution of colloid chemists Bungenberg de Jong and Saubert to the physiology of smell sprang from their work on "coacervates". Coacervates are colloid solutions, whose solvent is generally a solution of salt in water, which have an internal coherence, so that they cannot be diluted, but exist in equilibrium with their solvent without mixing freely with it.

Coacervates of lipids have the property that when foreign substances, especially organic ones, are added, the concentration of the coacervate changes. This may occur with very small amounts of the added substance. Bungenberg de Jong and Saubert (1937) proposed in general terms that this phenomenon could have some connection with the sense of smell, but they had to be vague, in view of the sparse physiological data available at the time.

In the context of the above picture of olfactory physiology, a "revised coacervate hypothesis" can be formulated.

The sensitive membrane of the olfactory receptor cells is assumed to have regions where the membrane lipids take on a coacervate-like structure. When an odorant arrives at the membrane, it enters these regions and there causes the ratio between water, which is electrically conducting, and lipid, which is not, to change so that the ionic permeability changes.

The last step of this reasoning has been supported experimentally: if the concentration of a lipid coacervate is raised through the addition of an organic substance, its electrical conductivity falls (Sperber *in press*). The conductivity is, of course, a measure of the permeability of the coacervate to the ions present, in this case mainly sodium and chloride ions.

If the lipid coacervates are to serve as a membrane model, their structure is naturally of major interest. Little is known with certainty about this, but a good working hypothesis has been presented by Boofj (1949), according to which the lipid is distributed in the form of flat "sandwich" micelles, essentially pieces of lipid bilayer. The negative surface charge of the micelles is assumed to be nearly neutralized by positive counterions which are unevenly distributed, so that the micelles can come into loose contact with each other at points where their charge is completely neutralized by counterions, but are still kept apart by the electrostatic repulsion between regions where the charge is not completely neutralized. As occlusion water will then be locked between the micelles, this picture explains how a considerable but limited amount of water can be kept in the coacervate.

Even less is known about the mechanism by which foreign molecules influence the conductivity of the coacervate, and available hypotheses are unconvincing. See (Sperber *in press*) for a discussion of this problem.

In order to gain more insight into the possibilities of the model, a technically suitable lipid coacervate has been developed and its properties investigated (Sperber *in press*). A



Fig. 2. This figure is intended to give an idea of how small buds on the membrane, about 20 nm thick, could contain relatively complex, coacervate-like structures. It is not to be interpreted as showing actual data. The rectangles denote lecithin molecules (about 2.5 nm in length). The lines denote protein.

The coacervants were uncharged organic compounds with a polar group (alcohol, ketone, ether or ester) and a hydrocarbon chain with 4-8 carbon atoms. There are many biological compounds of this type, but they are volatile. It is possible that the receptor cells could synthesize such compounds rapidly enough to keep the receptor membrane in a steady state, but in cells that are specialized for close diffusional contact with the outside air it is not a tempting concept. A more attractive thought is that the role of the coacervant is taken over by amino acid residues of the membrane proteins. An amino acid residue with its peptide group is in fact quite similar to the coacervants, except that its mobility is reduced by its being a member of a polypeptide chain. This difference may not be important. Double coacervates containing both proteins and lipids exist, although mainly with one-chain lipids rather than lecithin. According to Pinkhurst (1949), one kind of double coacervate with gelatin and lauryl sulphate probably has one lauryl sulphate molecule bound to each peptide 'link' in the gelatin.

Therefore, as an extension of the model, the coacervate-like structure in the receptor membrane is assumed to be a complex between lipid and protein, but retaining the essential characteristics of the protein-free coacervates used in the experiments. This assumption will be used in the following discussion.

Another question is whether there will be space enough in the membrane for a coacervate-like structure. At present, it cannot be determined how small a volume of coacervate will retain the macroscopic properties. It is conceivable that the forces determining changes in coacervates may operate on a mere pore or rift in the membrane. Even a more complex structure, such as sketched in Fig. 2 would only constitute moderate buds on the membrane. In principle, micellar structures may well occur in membranes: this has been discussed by Lucy (1970).

The membrane potential change

Gesseland *et al.* (1963) found that the response of a receptor cell to an odour was in most cases an excitation, but inhibition was not uncommon. In general, this has been confirmed by later investigators (for references, see Sensitivity patterns). There is, however, some doubt about the physiological relevance of inhibition (Duchamp *et al.* 1974). Also in many cases, inhibition may have been an artifact caused by withdrawal of background odour.

TABLE I Traits in the dependence of coacervate sensitivity patterns on the coacervant.

Trait No	Coacervant	Coacervate particularly		Comments
		sensitive to	insensitive to	
I	Any alcohol	Alcohols		
II	Benzyl alcohol		Hexane	
III	Heptanone		Ether	
IV	Heptanone		Ethyl propionate	Compared to less coacervant as ethyl propionate or ether
V	Benzyl alcohol	Hexanone		
VI	Heptanone or octanol or hexenol	Ethyl benzoate and anisole and toluene		

Data

For a full presentation see Sperber (in press)

It was found that when an additive is included in a coacervate, the conductivity of the latter falls roughly in proportion to the amount of additive. The proportionality factor, the "sensitivity" depends on both additive and coacervant.

This dependence was investigated for 9 coacervants and 20 additives. Some of the results are shown in Fig. 1. All the coacervates tended to be more sensitive to additives with long hydrocarbon chains. Beyond this simple rule, the dependence was very complicated. 5 relatively simple and distinctive traits could be discerned in the material. These are presented in Table I.

The optical properties and chemical composition of the coacervates were consistent with the structure pictured by Boolj. The dye diffusion experiments, however, suggested that the lipids may form a continuous, though not static, network rather than micelles.

Comparison between the coacervate model and biological data

In this section, the biological relevance of the revised coacervate model for olfactory transduction will be discussed although sufficient information is not yet available to make a complete evaluation of the model.

Conditions for the existence of coacervates in cells

At normal temperatures, the coacervates form spontaneously from their constituents. Of the constituents, water and salt are present in living cells, and lecithin is abundant in the olfactory epithelium (Koyama *et al.* 1971), though not exactly identical with egg lecithin.

Dodecyl benzene sulphonate is a nonbiological compound, but it was chosen for technical reasons, and a coacervate will still form if it is exchanged for another anionic surfactant, such as palmitate or lauryl sulphate or if it is excluded altogether (Sperber 1973). Negatively charged lipids, such as fatty acids, cephalin or phosphatidic acid should be able to take its place in a cell membrane.

volve the presence of two different types of conservate in the same membrane, with different selectivity and different sensitivity patterns to odorants. On the other hand, it is easy to show that *e.g.* ethanol causes an increase in the conductivity of the conservate. Also, other lipid conservates, *e.g.* oleate conservates, often react to additives with a decrease in conductivity (Bungenberg de Jong *et al.* 1938), which ought to mean an increase in conductivity in a conservate especially the case if the additive is of low molecular weight or is used in comparatively large amounts (most single-cell recordings have been obtained using rather powerful stimuli).

Sensitivity patterns

There are several investigations published where recordings have been made from a number of single receptor cells, while they are stimulated with a number of different odorants. Except for insect "specialist" receptors, such experiments yield bewildering results (Boeckh *et al.* 1965 Kay 1971 Sess 1972, Holley 1974, Giachetti and MacLeod 1973 Duchamp *et al.* 1974, Holley *et al.* 1974, Mason and Friggi 1974, Mustaparta 1975 For further references on vertebrates, see Getchell 1973). The cells have, as a rule, a rather low selectivity and may show similar reactions to odorants that are both chemically and organoleptically similar. Two cells with identical sensitivity patterns are very difficult to find. Of several attempts to find at least rudimentary order in such results, the most convincing is that of Duchamp *et al.* (1974) on the frog. They found that any single cell tended to give similar responses to all the aromatic compounds among the stimuli.

Some of the observed differences between cells may be caused by factors outside the cells. According to Mozell (1964), a kind of chromatography may occur on the olfactory mucosa causing different odorants to be concentrated in different parts of the mucosa, so that identical cells in different positions will be expected to show different sensitivity patterns. Also, the position of the cilia in the mucus covering the epithelium should be important. A cell whose cilia lie on the surface of the mucus will be reached by all odorants, while a cell whose cilia lie deep in the mucus, for example a freshly developing cell such as observed by Grunadai and Metcalf (1971), will be reached mainly by odorants with high water solubility.

It is, however, by no means possible to explain all the results in this way and it is clear that the sensitivity pattern of the receptor membrane itself can vary considerably between cells. This is very likely the basis of the ability to distinguish between smells. It is very important for a hypothesis of receptor cell function to explain the variability of sensitivity patterns, and both theories mentioned earlier make a point of it. Rosenberg *et al.* (1966) showed directly that different carotenoids have very different sensitivity patterns. In the "penetration and puncturing theory" there is not such clear evidence, but it is assumed that the sensitivity pattern of a membrane will depend on its viscosity (Davies 1970).

The conservate hypothesis can also explain the variability in sensitivity patterns, since it has been clearly shown (Sperber *in press*) (see also Fig. 1) that conservates show different sensitivity patterns, depending on which conservant is used. Thus, if the conservate-like structures in two cells contain different amino acids, the cells will be expected to have different sensitivity patterns. There is no definite limit to the variation possibilities, though it seems that the conservates so far investigated do not differ as much as receptor cells do.

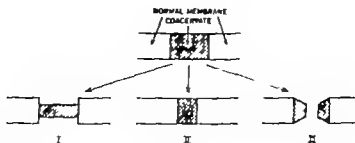


Fig. 3 If coacervate-like structures in a membrane shrink under the influence of odorants, they can contribute, depending on mechanical factors, (I) a permeability increase because of membrane thinning, (II) a permeability decrease, because of channel narrowing, (III) a large permeability increase, because of pore formation.

It is not clear what changes in the permeability of the receptor membrane cause the generator potentials. Attempts have been made to investigate them by ion substitution techniques (Takagi *et al.* 1966, 1968, 1969), but the results are doubtful, because the experiments were of such long duration that the thin cilia of the receptor cells may have been considerably damaged by the solutions. Also, single cells were not studied, but the EOG which may contain several components.

Thus, many ion species may be involved in the generator potential. To simplify the reasoning, however, the following discussion will concern only the roles of sodium and potassium ions.

In other cases, generator potentials are usually caused by an increased permeability to some ion, but not without exception (Tomita 1970). Therefore, the coacervates used in the experiments could be involved in generator potentials in spite of their strong tendency to decreased conductivity in response to additives. Also, they may cause either depolarizing or hyperpolarizing generator potentials, depending on the properties of the normal (non-coacervate) part of the membrane. If the resting potential is assumed to be determined by the relation between sodium and potassium permeabilities, and the coacervate parts are more potassium selective than the normal membrane, an overall permeability decrease in the coacervate parts will result in a decreased potassium selectivity of the whole membrane and a depolarizing generator potential. Conversely, if the coacervate parts are less potassium selective than the normal membrane, a hyperpolarizing generator potential will result. Of course, if a selectivity change in the coacervate itself were to accompany the overall permeability decrease, a generator potential would result irrespective of the properties of the normal membrane.

In this connection it should be pointed out that odorants cause not only the conductivity, but also the volume of lipid coacervates to decrease, especially in the more dilute coacervates. If such a volume decrease occurs in coacervate-like structures in a membrane, it may lead to various changes in the permeability depending on mechanical parameters (Fig. 3).

In terms of the coacervate model it is a little more difficult to account for the fact that one cell may show both excitatory and inhibitory responses to different odours unless the inhibitory responses are artifacts or caused by a different mechanism. If the coacervate can react to an odorant only with a decrease in permeability (as in (Sperber *in press*)), one must

$$E = 61 \log \frac{(25+2)4 + (1+2)145}{(25+2)155 + (1+2)12} = -54.3 \text{ mV}$$

the permeabilities of the coacervate part are decreased by 14% the membrane potential will be

$$E = 61 \log \frac{(25+1.72)4 + (1+1.72)145}{(25+1.72)155 + (1+1.72)12} = -56.2 \text{ mV}$$

a change by about 3.5%. It does not seem unreasonable that this could cause an appreciable change in the spike frequency of the cell.

A rough estimate of the amount of odorant required for the transduction (the total amount required will be considerably larger because the normal membrane will also take up odorant) can be calculated as follows:

If the normal membrane has a K⁺ conductance of 5 S/m (2000 $\Omega \text{ cm}^2$), then, under the foregoing assumptions, its Na⁺ conductance will be 0.2 S/m² and the K⁺ and Na⁺ conductance of the coacervate parts will be 0.4 S/m each and the total conductance of the coacervate parts 0.8 S per m membrane. If the coacervate parts have a conductivity of 1 S/m and are assumed to be $20 \cdot 10^{-6}$ m thick (cf. Fig. 2), 1 m of coacervate membrane will have a conductance of

$$\frac{1}{20} \frac{1}{10^{-6}} = 5 \cdot 10^7 \text{ S.}$$

If the coacervate parts of 1 m of membrane have a conductance of only 0.8 S, this means that

$$\frac{0.8}{5 \cdot 10^7} = 1.6 \cdot 10^{-8}$$

of the membrane area consists of coacervate. At threshold, only the most superficially exposed cells on the epithelium will contain appreciable amounts of odorant, since their membrane lipids act as a sink for the odorant molecules. Their membranes will have an area comparable to that of the regio olfactoria, i.e. of the order of 10^{-3} m² of which $1.6 \cdot 10^{-8} \cdot 10^{-3} = 1.6 \cdot 10^{-11}$ m² is coacervate, and contain $1.6 \cdot 10^{-11} \cdot 20 \cdot 10^{-6} = 3.2 \cdot 10^{-17}$ m³ of coacervate. As the coacervate contains about 10^{20} lipid, there will be $3.2 \cdot 10^{-17} \cdot 10^{20} = 3.2 \cdot 10^3$ cm³ lipid, weighing about $3.2 \cdot 10^{-14}$ g, in turn containing 0.06 mmol of odorant per g, the coacervate parts will contain $3.2 \cdot 10^{-14} \cdot 0.06 = 1.9 \cdot 10^{-15}$ mmol, i.e. of the order of 10^9 odorant molecules.

The location of the interaction

Some attempts have been made to show that some specific kind of molecules in the membrane act as acceptors for the odorant molecules. Odorants have been shown to interact with various kinds of molecules, but it is still not clear which interactions are essential and which are accidental. There seems, however, to be a general feeling that protein molecules are the most likely acceptors.

Spectrometric methods have shown that odorants cause conformation changes in both lipids (Dodd 1971) and proteins (Ash 1969) in the olfactory epithelium. In neither case have the changes been shown to be connected with the transduction process.

In view of the grouping of aromatic odorants found by Duchamp *et al.*, mentioned above it is interesting to note that coacervates where the coacervant has a comparatively long hydrocarbon chain seem to have an increased sensitivity towards aromatic additives (the last is in Table 1). At the present stage it is hardly meaningful to try any more detailed comparisons between the sensitivity patterns of receptor cells and coacervates.

Degree of sensitivity

It is very difficult to calculate whether the sensitivity of the coacervates to odorants is high enough to explain the high sensitivity of the sense of smell. About such factors as turbulence in the nasal passages, ion distribution and permeability of the receptor membrane and its sensitivity of the action-potential generating mechanism there is at least room for reasonable guesses. The most troublesome problem is that the receptor cells as a rule are spontaneously active and therefore do not have a definite threshold. Instead, the olfactory threshold will ultimately be determined by the ability of the brain to register minute changes in the average firing frequency of the receptors. About this ability we know hardly anything. It may be exceeded the corresponding ability of the experimenter studying single cells, since the receptors converge strongly on the higher-order neurons. In spite of these difficulties, the following argument attempts to show that the sensitivity of the coacervates may well suffice.

Choosing decanol as an example, we find the biological threshold for man given as $3.6 \cdot 10^{-10}$ molecules per cm³ of air (Davies and Taylor 1957). According to the same source, the partition coefficient between the lipid-water interface and air is 10^{-10} . Thus the threshold concentration in air corresponds to an equilibrium concentration of $3.6 \cdot 10^{-20}$ molecules per cm³ in the membrane. As the lipids have about the same density as water this corresponds to about 0.6 mmol of decanol per g lipid. It is doubtful whether there will be time under normal conditions for equilibrium to be reached, but if we assume that at least 10^{-10} of equilibrium is reached in the membrane of the most superficial cilia this means that 0.06 mmol per g lipid are needed for stimulation.

The coacervate where the coacervant was ether showed a sensitivity towards decanol of about 5 kS/mol m.¹ Since the coacervate contained about 0.45 g of lipid (lecithin + surfactant) this means that a concentration of 0.06 mmol per g lipid causes a decrease in conductivity of $0.06 \cdot 0.45 \cdot 5 = 0.135$ S/m. This means that the conductivity of the coacervate decreases by about 14%, since the coacervate without decanol had a conductivity of nearly 1 S/m.

Assume that the membrane potential of the receptor membrane is determined by its permeability to sodium and potassium ions, which have normal concentrations ($\text{Na}^+ = 145$ mM, $\text{Na}_i = 12$ mM, $\text{K}^+ = 4$ mM, $\text{K}_i = 155$ mM). Assume that the normal part of the membrane is 25 times more permeable to K^+ than to Na^+ while the coacervate parts are nonselective. Assume that the area and permeability of the coacervate parts are such that they contribute to the total membrane sodium and potassium permeabilities that are twice the sodium permeability of the normal part. Then by the Goldman equation the potential of the unstimulated membrane is

$$S \text{ (volts)} = 0.059 \log \frac{P_{\text{K}} + 2P_{\text{Na}}}{P_{\text{K}}}$$

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Most successful are the striking experiments of Gatchell and Gesteland (1972). They show that the response to odours, as measured by the EOG, was destroyed when the olfactory epithelium was treated with the protein reagent, *n*-ethyl maleimide, except the response to odours that were present in high concentration during the treatment. These results strongly indicate that proteins are involved in the specific processes of olfaction. They do not, however, prove that the primary interaction of the odorants is with protein. If the odorants interact primarily with the lipid part of a lipo-protein complex, this may protect the protein part against the reagent. It is known that agents interacting with the lipid part of lipo-proteins may alter the reactivity of the protein part with protein reagents (Godin and Wan Ng 1973).

Thus, the model of olfactory transduction occurring primarily with the lipids in a lipid-protein double coacervate seems consistent with these experiments.

The ability of the sense of smell to distinguish between enantiomers, best established in the case of carvone, is often regarded as evidence for odorant interaction with proteins. However, phospholipids also show enantiomerism (the β -carbon in the glycerol residue is asymmetric) so there is in principle no reason why lipid systems could not have the same ability. A rigid receptor site, such as may occur in proteins, would be expected to give a better discrimination but Friedman and Miller (1971) conclude that carvone probably does not interact with such a site. Also the results of Lettner *et al.* (1971) suggest that the carvone enantiomers are not drastically different physiologically as the experimental subjects confused them in about 20% of the cases.

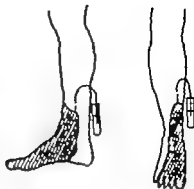
Conclusions

It seems that the olfactory receptor membrane may well contain regions where its lipids are organized essentially as in lipid-protein coacervates. This model can in principle explain much of the available physiological data. In particular it explains how odorants can cause permeability changes in the membrane and how different receptor cells can have different sensitivity patterns towards odorants. Both physicochemical data about the model and physiological data about the cells are insufficient to allow a more detailed evaluation of the model. As further experimental work is possible along both lines, there is hope for future improvement.

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Fig. 1 Leg lymph cannulation. Hatched field indicates area used by the cannulated lymph vessel. The tissues drained skin, subcutaneous tissue and presumably muscular tissue.



on of cells, proteins and enzymes was determined, and its dependence on the body position, muscular activity and various procedures known to increase blood capillary filtration assessed. The present paper deals with the variation in lymph flow and protein and enzyme concentration during night rest and normal daily activities.

Material and Methods

Healthy male volunteers aged 19 to 27 were studied. A superficial lymph vessel was cannulated under sterile conditions on both legs about 15 cm above the ankle, according to the technique previously described (Engset *et al.* 1973). The lymph vessel drained skin, subcutaneous and perivascular tissues of the foot of at least approximately as defined in Fig. 1. This cannulation failed on one leg. Lymph was collected into sealed test tubes 10 or 15 cm above the cannulation site—depending on the volume—and flow measured.

For protein and enzyme studies, lymph samples were spun down to remove cells. The supernatant was immediately stored at -20°C in sealed test tubes until it could be analyzed. Blood samples were taken from the cubital vein at the end of lymph sampling periods, and parallel studies carried out. Total protein concentration was determined by Biuret method, electrophoresis was performed on cellulose acetate strips. Lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activity were determined with the Scandinavian recommended methods (see reference) using the LKB reaction rate analyzer. Lymph protein and enzyme output and lymph vessel concentrations were calculated. Because of significant variations in basic parameters between the individuals and between legs of the same individual, due mainly to different topography and caliber of the cannulated vessels, and because from day to day variation on the same leg the results have been presented as percentage of the night value from the same leg over the same 24 h period. The range of protein and enzyme concentration and lymph flow has also been presented in bar charts.

Experimental schedule

Studies were started on the third day after cannulation. The 24 h period was divided into 9 intervals of different duration devoted to various activities.

- | | |
|-------------|---|
| 23.30–04.30 | Night rest. |
| 04.30–08.00 | Getting up, morning activities (morning toilet, breakfast and journey to the hospital). |
| 08.00–09.00 | Lying in the horizontal position. |
| 09.00–12.00 | Fast sitting. |
| 12.00–13.00 | Lying in the horizontal position. |
| 13.00–15.00 | Various procedures increasing capillary filtration. |
| 15.00–16.00 | Lying in the horizontal position. |
| 16.00–19.00 | Ordinary afternoon activities. |
| 19.00–23.30 | Ordinary evening activities. |

From the Laboratory of Hematology and Lymphology, Norsk Hydro's Institute for Cancer Research, the Norwegian Radium Hospital and Clinical Laboratory, the Norwegian Radium Hospital, Montebello, Oslo, Norway and the Surgical Research and Transplantation Laboratory, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

Twenty Four Hour Variation in Flow and Composition of Leg Lymph in Normal Men

By

A. ENGESET, W. OLSZEWSKI, P. M. JÄGER, J. SOKOŁOWSKI and L. THEODORSEN

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Abstract

ENGESET A., W. OLSZEWSKI, P. M. JÄGER, J. SOKOŁOWSKI and L. THEODORSEN. *The 24 hour variation in flow and composition of leg lymph in normal men*. Acta physiol. scand. 1977 99 140-148.

The twenty-four hour variation in concentration and output of total protein, lactate dehydrogenase and alkaline phosphatase has been studied in the peripheral lymph from the legs of 5 healthy volunteers over a period of 11 days. The highest concentration of these proteins was found in lymph collected during the first two hours after night's rest. During the day a continuous decrease in concentration occurred, and a direct correlation to lymph flow. The variation in lymph protein concentration between early morning and late day was about 40%, lactate dehydrogenase and alkaline phosphatase about 90 and 45% respectively.

Key words: Activity cycles, body fluids, capillary permeability, circadian rhythm, enzymes, extracellular fluid, lymph, lymphedema, proteins.

The flow rate and composition of peripheral lymph depend *inter alia* on the hydrostatic and oncotic pressures in the intravascular and extravascular compartments (Starling 1891, Drinker and Field 1933, Guyton 1963, Courtice 1971), regional permeability of blood capillaries (Mayerson *et al.* 1960) and initial lymphatics (Casley-Smith 1970), and on molecular weight and size of the various protein components in blood and tissue fluid (Pappenheimer *et al.* 1951, Garlick and Renkin 1970, Grotte 1956, Renkin 1964). Variations in lymph flow and composition will occur depending on the functional status and position of the tissue from where lymph is collected. Preliminary studies of the 24 h variation of leg lymph flow and composition in patients with malignancies revealed a pattern of variation which to some extent was in contradiction to that found in animal experiments (Jäger 1975). The findings were confirmed in the present study of peripheral lymph in healthy male volunteers, followed systematically throughout several days. The 24 h variation in the lymph flow, concentration

L. Total protein concentration and enzyme activity in leg lymph from 5 healthy volunteers. Mean of 5 days for each leg and for all men \pm S.E.

Leg	Total protein g/100 ml		Alkaline phosphatase U/l		Lactate dehydrogenase U/l	
	Night	24-hr period	Night	24-hr period	Night	24-hr period
L	1.70	1.80	16	18	42	57
R	2.12	1.97	26	18	82	63
R	1.44	1.62	20	15	83	34
L	2.03	1.85	30	20	48	33
R	2.65	1.80	25	18	63	43
L	2.43	2.14	33	26	92	57
R	2.40	1.99	35	26	43	26
L	2.20	2.11	36	28	74	84
R	2.75	2.46	48	37	95	72
n	2.16	1.97	29.9	22.9	65.07	49.22
	0.12	0.083	3.19	2.32	6.96	5.13

lymph protein concentration and output

lymph total protein concentration was in the range of 1.37 to 2.98 g/100 ml. The mean for concentration was 2.14 ± 0.1 (1 S.E.) g/100 ml. The concentration varied between individuals and between the two legs of the same man. All legs showed the same pattern of relation throughout the 24 h period. There was an increase of about 20% after getting up the morning, then a steady drop towards the end of the day to values below the night level (Table I, Fig. 2). No direct correlation between the flow and lymph protein concentration was found throughout the day (Fig. 3). Increase in flow after assuming an upright position the morning was accompanied by the highest increase in protein concentration. Then

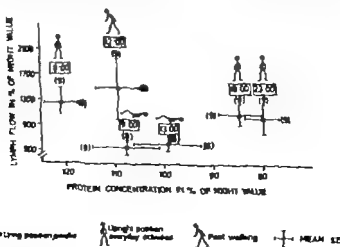


Fig. 3. 24-hour relationship between lymph flow and lymph protein concentration, both expressed as percent of the night value. Mean values from 9 legs for 5 days \pm S.E.

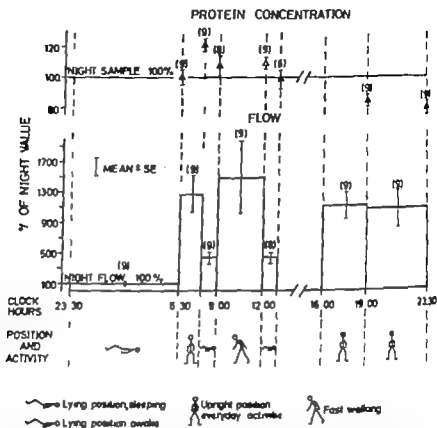


Fig. 2. 24-hour variation in leg lymph protein concentration and lymph flow expressed as % of night values from the same leg over the same 24-hour period. Mean values from 9 legs for 5 days \pm one standard error. Symbols indicate the position and activity during lymph sampling. Lymph collected between 13.00 and 16.00 is excluded because of special experiments during this period.

Lymph was collected continuously into test tubes replaced at the end of each sampling period, sealed and the content of each tube, comprising all lymph sampled during the preceding interval, was analysed separately. Lymph volume and protein concentration were determined in all samples. The enzyme concentration was not analyzed in the samples collected after the 60-minute periods in the horizontal position because the samples were too small.

Blood samples were taken at the following times: 08.00, 09.00, 12.00, 13.00 and 16.00 hours.

Results

Lymph flow

The flow was in the range of 0.01 to 0.55 ml/h throughout the observation period. There were major individual variations and also variation between the two legs of the same man as well as from day to day variations. The mean night flow was 0.24 ± 0.056 (1 S.E.) ml/h. There was a mean 13-fold increase in flow after getting up in the morning (Fig. 2). The flow dropped during the 60-minute periods in the horizontal position, but the mean was still almost 5 times higher than during night rest. Fast walking increased the flow to about 15 times the night values. During the evening the flow was about 10 times higher than the night flow.

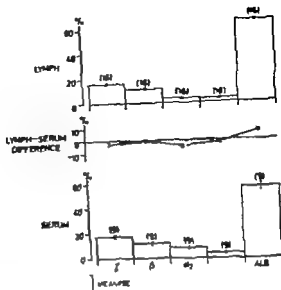


Fig. 3. Leg lymph and serum electro-osmotic patterns. Mean values from 5 \pm S.E. (9 legs examined). The paired *t* test used to measure the difference. Samples taken after 3 h of fast sleep.

Discussion

The main finding has been a marked 24-hour variation in lymph protein and enzyme concentration and output without any direct correlation between lymph flow and protein concentration. This differs with what has been observed in the peripheral lymph of running dogs (White, Field and Drinker 1933; Garlick and Renkin 1970; Joyner *et al.* 1973; Sævi 1964). They found according to Drinker's theory (1933) on lymph formation, that the protein concentration was inversely related to lymph flow when capillary permeability was constant. In the present study there was always a low lymph flow during the night, and the protein and enzyme concentration was relatively high. After getting up in the morning, both lymph flow and concentration increased significantly. During subsequent fast walking the protein concentration was maintained at a relatively high level regardless of a high lymph flow. Finally lymph protein and enzyme concentration and lymph to serum ratio revealed a tendency to decrease in the course of the day. We consider the following mechanisms most likely responsible for the observed variations in lymph flow and composition.

Night lymph

1. In the horizontal position during sleep there is a low capillary hydrostatic pressure (Landis 1930) with decreased filtration and may be also increased reabsorption of excess water and solutes from the tissue fluid. Furthermore, there is a reduced muscular activity which reduces the propulsion of lymph in the collecting trunks (Taylor *et al.* 1973). For this reason the lymph flow during the night will be low.
2. There is, however, all the time a diffusion of proteins and other large molecules through out the walls of the exchange vessels (Garlick and Renkin 1970). All this will result in a high protein concentration of the interstitial fluid. The lymph sampled in the vials throughout the night will, however, be a mixture of

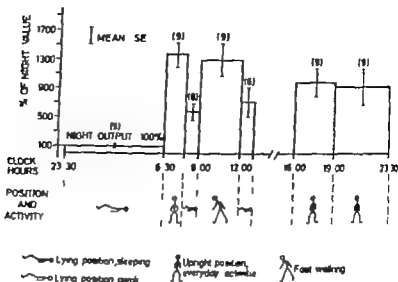


Fig. 4. 24-hour variation in leg lymph protein output expressed as % of night value from the same 24 h period. Mean values from 9 legs for 5 days \pm 1 S.E.

during fast walking, protein concentration remained high despite a 15-fold increase in lymph flow.

Lymph protein output per hour reached the highest values in the first morning sample as a result of an abundant flow of concentrated lymph (Fig. 4). During fast walking it was an average 13 times higher than during the night. During rest periods it decreased to a half the walking values.

Serum protein concentration was not studied early in the morning (before getting up) and during the evening. During the day the serum protein concentration dropped by a 5% following 60 min of rest in the horizontal position, and increased slightly after walking (Fig. 6).

Electrophoresis (of samples obtained after 3 hours of fast walking) revealed a 7% higher albumin and a 40% lower α -2-globulin concentration in lymph than in serum (Fig. 5).

Enzymes

The enzyme concentration followed the protein pattern during the observation period (Fig. 6). The range of AP activity was 12 to 53 U/l. The mean night AP concentration in lymph was 31.7 ± 3.8 (1 S.E.) U/l. The first morning lymph sample had 20% higher activity than the night sample; from then on a steady decrease was observed. The LDH activity was within a range of 5 to 157 U/l. The mean night value of LDH in lymph was 79.8 ± 9.8 (1 S.E.) U/l. It was 52% higher in the first morning sample than during the night. It decreased gradually towards the end of the day down to 50% of the night value in the last evening sample (Table I, Fig. 6).

Serum LDH and AP concentration revealed the same type of fluctuation as the lymph values. Serum protein value with an average of 5% fall during rest in the horizontal position (Fig. 6). A decrease in the ratio lymph/serum of LDH and AP was observed.

face may take place due to opening of capillaries, closed at low pressure, and stretching capillaries which may evoke "stretch pore effect" (Shirley *et al* 1957) increasing lymphation.

lymph collected during fast walking

the washout of proteins accumulated during the night is probably a time-consuming process. It may explain why a high-protein concentration was still maintained during fast walking the morning, despite a high lymph flow. In addition, increased capillary filtration of proteins in active tissue may play some role.

lymph collected during short rest periods in the horizontal position

The relatively high mean lymph flow and lymph protein concentration during the rest period between 8-9 and 12-13 hours compared with the night period was most likely caused by the postexercise hyperemia following muscular activity with most capillaries open, thus high filtration area.

lymph sampled towards the end of the day

The mechanism behind the continuous decrease in protein concentration of lymph during a day despite constant or somewhat reduced lymph flow is difficult to explain. It might be that the washing out of the proteins accumulated in the tissue space during the night takes long time that it will influence the protein concentration of lymph produced in the evening.

Immunoglobulins

Higher concentration of albumin and a lower concentration of alpha-2-globulin were found in lymph compared with serum. The alpha-2-macroglobulin accounts for the bulk of the alpha₂-band in the serum. This has a high molecular weight. The finding is therefore in accordance with the molecular sieving theory.

Enzymes

Two different enzymes were chosen for parallel studies. These were lactate dehydrogenase which can be locally produced by the drained tissue, and alkaline phosphatase originating from other tissues and easily equilibrated in body fluids. The variation of concentration of enzymes in the serum followed that of the protein, with a fall during rest (Fig. 6). The lymph concentration of AP followed the same pattern as lymph concentration of proteins. The concentration of LDH is somewhat different, the curve is steeper and there is a steady fall until the last sample taken late in the evening. The reason for the continuous drop in LDH throughout the evening might be that the amount of locally-produced LDH (muscle enzymes) decreases with reduced muscle activity.

To conclude, a 24 h variation in concentration of protein and enzymes in peripheral lymph has been found. The mechanism behind this is discussed without taking into consideration parameters such as diurnal variation in cell metabolism, hormone status and capillary filtration. Before a more sophisticated discussion can be fruitful, these parameters have to be studied and also the direct effect of the erect and supine position. Such experiments are

From the Laboratory of Hematology and Lymphology Norsk Hydro's Institute for Cancer Research, the Norwegian Radium Hospital and Clinical Laboratory of Norwegian Radium Hospital, Moncebello, Oslo, Norway and the Surgical Research and Transplantation Laboratory Medical Research Center Polish Academy of Sciences, Warsaw Poland

Flow and Composition of Leg Lymph in Normal Men during Venous Stasis, Muscular Activity and Local Hyperthermia

By

W. OUSZCZAK, A. ENGESBET, P. M. JACOB, J. SOKOLOWSKI and L. THEODORSEN

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Abstract

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Changes in venous leg lymph protein concentration, osmotic, and lymph flow/lymph protein concentration relationship, as well as lactate dehydrogenase and alkaline phosphatase activity were followed during procedures known to increase capillary filtration in venous stasis, muscular exercise and warming tissues. Lymph flow increased by 83% during two hour ergometer cycling, and by 117% during two hours after foot bath. During two hour period of venous stasis lymph flow dropped by 50% and by 44% after foot warming. An inverse relationship between the lymph flow rate and lymph protein concentration is found. Lymph enzymes followed the same pattern of changes as total protein.

Keywords: Activity cycles, body fluids, capillary permeability, circadian rhythm, enzymes, extracellular fluid, lymph, lymphoedema, proteins.

The rate of formation, flow and composition of lymph in human leg depends mainly on the position of the extremity, its muscular activity and ambient temperature. Change of position from the horizontal to the upright is accompanied by a rise in venous hydrostatic pressure (Henry *et al.* 1955). Walking causes an increase in blood flow through the leg tissues and muscle pump promotes lymph flow (Taylor *et al.* 1973). Ambient temperature may influence lymph formation by dilatation or constriction of skin microcirculation (Henry *et al.* 1955). All the three described physical factors may to different degrees, affect lymph volume, protein concentration and lymph/serum protein ratio. The knowledge of these differences would be important for understanding of the mechanism of interstitial fluid volume regulation in human leg.

in progress. A practical consequence of the observed 24 h variation is that data on perifer lymph flow and composition to be informative must always be correlated with the same conditions.

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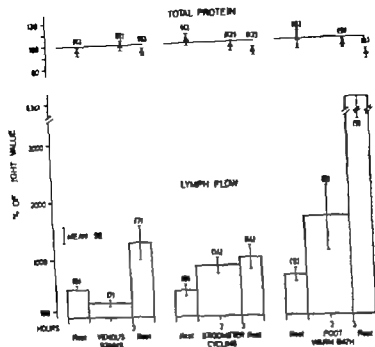


Fig. 1. Lymph flow and protein concentration in the leg before, during, and after leg venous stasis, ergometer cycling, and warm water bath. Values from 5 men expressed in percentage as means of right values in the same leg over the same 24 h period \pm 1 S.E.

Fig. 3) Most pronounced changes were seen in the rest period after warm water bath. Lymph flow increased by 273% reaching values 63 times higher than the right samples. Protein concentration fell by 9.3%. Protein output was doubled and 33 times higher than the right value. LDH and AP dropped by 23.4% and 15.5% respectively. The increase in flow is accompanied by decrease in protein concentration (Fig. 4).

Discussion

The purpose of the present study was to follow the protein and enzyme transport in the interstitial fluid and lymph in three experimental situations, to some extent different in the mechanism of increase in capillary filtration. In the first, venous hydrostatic pressure was increased in leg using a sphygmomanometer cuff. This elevated the capillary filtration pressure. In the second, blood flow in the leg was stimulated by strenuous ergometer cycling. This opened muscle and also skin capillaries and increased capillary filtration surface area. In the third, leg tissues were warmed to 41°C, which produced maximum opening of skin capillaries and their dilatation with subsequent increase of filtration area and enhanced permeability of the capillary wall (Garlick and Renkin 1970, Arthurson *et al.* 1969, Courtois and Sabene 1966).

A decrease of lymph flow was found during venous stasis, a slight increase during ergo-

In the present study changes in leg lymph protein concentration and output, lymph protein concentration relationship and lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activity were observed during leg venous stasis, ergometer cycling and warm of the foot.

Materials and Methods

The present investigations were carried out on the same group of 5 young men as in the previous (Engset *et al.* 1976). Each had a leg superficial lymphatic cannulated and lymph collected. Details of lymph collection method were described earlier (Engset *et al.* 1973). The collected lymph was from the skin, subcutaneous tissue and muscular fascia of the anterior and medial aspects of foot and part of the leg. Investigations were carried out between 1 p.m. and 3 p.m. preceded and followed by 1 h of rest in the horizontal position. Only one type of experiment was performed on each individual each day. In group 1 a sphygmomanometer cuff was placed on the thigh and inflated to a pressure of 50 mmHg. In group 2, ergometer cycling was performed at a speed of 30 km/h with 2 kg load. In group 3, both legs were immersed in a water bath with a constant temperature of 41°C. Lymph was collected continuously into test tubes replaced at the end of each experimental and rest period. The content of each tube, containing all lymph sampled during the preceding interval, was analyzed separately. Lymph flow, lymph total protein concentration and output, LDH and AP activity were measured (as previously described by Engset *et al.* 1976). In the available blood samples the same protein and enzyme studies were performed. Because of significant differences in normal values of lymph flow, protein concentration and enzyme activity between the individuals and even legs of the same individual, and also from day to day variation (Engset *et al.* 1976) the results were presented in percent of the night values calculated for each leg every 4 h.

Results

Venous stasis

During leg venous stasis, lymph flow decreased by 50% compared with the rest period, and protein concentration increased by 4% (Fig. 1). Protein output fell by around 50% (Fig. 2). LDH and AP remained about the same level as at the beginning of the rest period (Fig. 3). Following release of the venous cuff lymph flow increased by 430%, at the same time protein concentration, LDH and AP activity decreased by 6%, 25% and 12% respectively. Protein output rose by 416% compared with the venous stasis period. Fig. 4 points to an inverse relationship between lymph flow and lymph protein concentration.

Ergometer cycling

Ergometer cycling increased lymph flow by 83% compared with the preceding rest period (Fig. 1). Protein concentration fell by 4%. Protein output was 52% higher than during the rest period (Fig. 2). LDH decreased slightly and AP remained unchanged when compared with the estimates from the beginning of the rest period (Fig. 3). There was a 15% increase in lymph flow in the period immediately after exercise and a decrease in protein concentration, LDH and AP by 5%, 3% and 9% respectively. There was a trend towards a decrease in lymph protein concentration with increase in lymph flow (Fig. 4).

Warm water bath

Warm water foot bath (41°C) brought about 117% increase in lymph flow (Fig. 1). Protein concentration dropped by 5% (Fig. 1). Protein output increased by 104% (Fig. 2). LDH and AP dropped by 28% and 17% when compared with the beginning of the rest period.

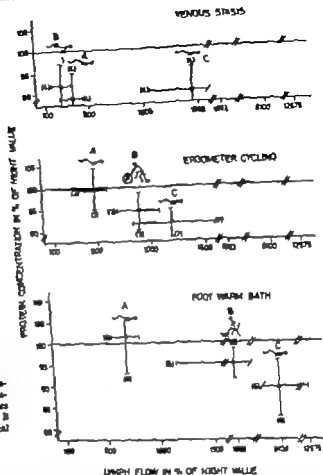


Fig. 4 Lymph total protein concentrations to lymph flow relationship during the three procedures shown in Fig. 1 A before, B during and C after experimental recovery

experiments performed on dogs (Seabo *et al.* 1963). The mechanism of this observation remains unclear. In the period of venous stasis the extremity was immobilized in order to deactivate the muscle pump as much as possible. The main force promoting lymph propulsion was then absent. In this situation capillary filtrate probably first of all filled the interstitial space, which caused a temporary drop in lymph flow. This may be in agreement with the observation that elevation of the venous pressure is followed by an increase in lymph flow (Garlick and Renkin 1970). Evacuation of the excess interstitial fluid began when the local tissue space pressure/volume values exceeded the safety limits (Guyton *et al.* 1971), which in the present study probably coincided with the release of the venous cuff.

During muscular exercise lymph flow was twice as high as during the preceding rest period. For interpretation of this observation one should remember that tissues under investigation were skin, subcutaneous tissue and muscular fascia. Only a small fraction of collected lymph could originate from muscles. Two factors should be considered as responsible for the increase in lymph flow. The first is the "muscular pump" which provides most of the driving force for the lymph flow in the limb superficial lymphatic system. The second

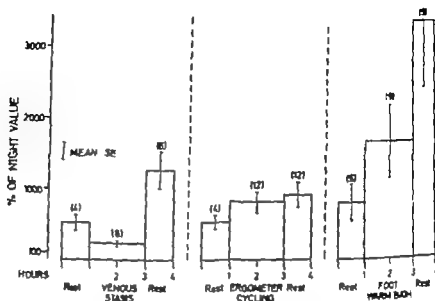


Fig. 2. Protein output in leg lymph during the same procedures as shown in Fig. 1

meter cycling, and an evident rise during warming of the foot. After completion of 1 experiment a high lymph flow was noted in all three groups. An inverse relationship between lymph flow and protein concentration was observed.

During venous obstruction a decrease in lymph flow in all individuals was observed despite evident clinical signs of venous stasis. The same finding was described by others

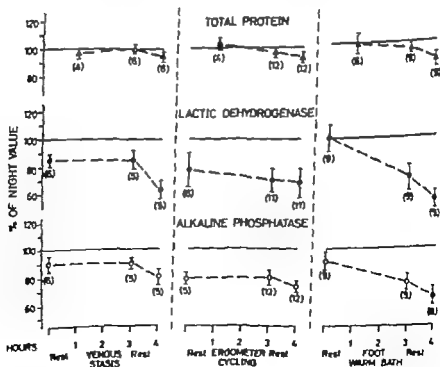


Fig. 3. Lactate dehydrogenase, alkaline phosphatase activity and total protein concentration during same procedures as shown in Fig. 1

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is an increase in skin lymph formation due to hyperemia accompanying increased blood flow. It has been found that strong muscular exercise of the lower limb in man produces a 2-3-fold rise in skin blood flow (Olszewski 1963).

High lymph flow during warming of the skin may be explained by increased capillary filtration due to opening of more capillaries and their dilatation followed by a rise in microvascular hydrostatic pressure (Landis 1930, Reaves *et al.* 1974). There is also an increased permeability of the capillary wall to protein (Arthurson *et al.* 1969, Courtois and Sabine 1966, Garlick and Renkin 1970) which has been attributed to the opening of large gaps between endothelial cells of postcapillary venules (Majno 1963). For high postwarming lymph flow a protracted effect of hyperthermia on the local blood flow regulation with vasodilatation and increased permeability and accumulation of large amounts of interstitial fluid in tissues may be responsible.

In all groups in the study an overall tendency although of a different degree, toward decrease in protein concentration with increase in lymph flow was noted. This would mainly be in agreement with the findings of other authors (Arthurson *et al.* 1969, Courtois and Sabine 1966, Garlick and Renkin 1970, Landis and Pappenheimer 1963, Sass 1964, Sjö *et al.* 1963, White *et al.* 1933). In the group with ergometer cycling a moderate rise in lymph flow and decrease in protein concentration may be accounted for by increase in capillary surface filtration area. No rise in capillary permeability would be expected.

In the group with venous obstruction, the proportionality between decrease in protein concentration and increase in lymph flow would indicate enlargement of capillary filtration area. In local hyperthermia, an extremely high rise in lymph flow was accompanied by rather small decrease in protein concentration, compared with the two former groups. Increased filtration area together with enhanced capillary permeability seem to be responsible for these findings.

In our previous study on 24 hour variation of leg lymph flow and composition, no relationship between lymph flow and protein concentration was found (Engeset *et al.* 1975). We attributed this to the time required to wash protein-rich lymph formed during rest before periods of activity out of the interstitial space and lymph vessels, thus a lag between formation and collection of lymph. Especially accumulation of protein during the night might have played a role here. The present experiments were performed in the afternoon when the protein-rich tissue fluid formed during the night has probably been washed out. Furthermore, high capillary filtration which accompanied the three present types of procedure permitted fast filling of interstitial space and promoted lymph flow so that flow of protein concentration could more accurately reflect the rate of lymph formation in tissues.

The lymph enzymes followed the same pattern of changes as did total protein. LDH and AP activity showed a tendency to decrease during all three types of experiment. This effect was most evident during the period immediately after procedures increasing capillary filtration, especially after warm water bath.

ids in skeletal muscle might contribute to a disturbance of the lipid metabolism during intake in intact animals and man. Previous studies on acetate metabolism in skeletal muscle have included investigation of the effect of acetate on the metabolism of exogenous glucose (Karlsson *et al.* 1976). The present report deals with the effect of acetate on the metabolism of palmitate, a long-chain fatty acid, in the perfused hind-quarter of the rat.

Material and methods

Animals

Spring-Dawley rats weighing 200–240 g, purchased from Antiknäs, Sollentuna, Sweden, were used. The rats were fed *ad libitum* on a standard small-animal diet obtained from Astra-Evo, Södertälje, Sweden, and were allowed free access to water prior to the experiments.

Media

All analytical grade laboratory reagents were obtained from E. Merck AG, Darmstadt, West Germany. Enzymes and co-enzymes were obtained from Boehringer-Mannheim, West Germany or from the Chemical Co. Inc., Los Angeles, USA. Solutions of benzobarbital (100 mg/ml) are freshly prepared from benzobarbital powder (Evgen, solution, Bayer AG, Leverkusen, West Germany). Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, England. Stock solutions of albumin (10%) were prepared in Krebs-Henseleit high-bicarbonate buffer (Krebs and Henseleit 1957) and dialysed at 4°C against a four-fold volume of the same buffer for 3–24 h.

^3H -acetate (38.5 mCi/mmol) and ^3H -palmitic acid (57.9 mCi/mmol) were purchased from the Chemical Centre, Amersham, England. Chemicals for measurement of radioactivity were obtained from Packard Instr. Co. Inc., USA and Koch-Light Laboratories Ltd, Buckinghamshire, England.

Perfusion technique and experimental procedures

Perfusion of rat hind-quarter perfused muscle was first described by Ruderman *et al.* (1971). All perfusions were done on skinned animals with the hind-legs at rest. The perfusion medium (170 ml) was the same as previously used at our laboratory (Karlsson *et al.* 1975) with an initial concentration of lactate of 1.9 mmol/l.

Glucose consumption and lactate production due to glycolysis in the bovine red cells during the perfusion period was negligible.

The perfusion was equilibrated by micro-perfusion injections of benzobarbital (25 mg/100 g b.wt.). The first 10 ml of the perfusate was discarded and the medium was then recycled. Substrates to be studied were added to the medium. Stock solutions of palmitate (33 mM) were freshly prepared by mixing 0.5 mmol of palmitic acid and 15 ml of water. These solutions were heated to 70°C for 15 min. Complete solution of the acid occurred after drop-wise addition of 1 M NaOH. The quantity of fatty acid solution to be added to the medium was mixed while still warm with pre-warmed dialysed 25% bovine albumin (prepared as the 10% solution) and this solution was added to the medium as a single dose. Acetate (1.5 M stock solution) was added as single doses followed by continuous infusion of acetate into the medium at a rate of 0.5 mmol/h/g muscle tissue as described previously (Karlsson *et al.* 1975). Samples of the medium for analysis of palmitate were taken at 15, 25, 40, 60 and 70 min. Samples for the analysis of acetate, lactate and pyruvate were usually taken at 15, 40 and 70 min. Metabolic rates ($\mu\text{mol/h/g wet weight}$) were calculated as changes in substrate concentration in the perfusate during the experimental period.

Analytical methods

Samples of the medium for analysis of the concentrations of lactate and pyruvate were added to 0.6 M CaCl₂. The extracts were neutralized with KOH and the precipitated KClO₄ was removed by centrifugation. The concentrations of lactate and pyruvate were measured in these extracts as described by Holmberg *et al.* (1979). Acetate and palmitate were determined in plasma according to Bergmeyer and Möller (1970) and Ixj and Ut (1965) respectively. A freshly prepared standard solution of acetate (2 mM) in buffer (4) was always run parallel to the plasma samples, when acetate was determined. Determination of the oxygen consumption by the perfused tissue was done as previously described (Karlsson *et al.* 1975). Oxygen measurements were usually done at 25, 35, 50 and 60 min.

Influence of Acetate on the Metabolism of Palmitate in the Perfused Hind Quarter of the Rat

By

N. KARLSSON, E. FELLENIUS and K. H. KJESLING

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Abstract

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The metabolism of $1\text{-}^{14}\text{C}$ -palmitate and its metabolic interaction with $\text{U-}^{14}\text{C}$ -acetate are studied in perfused hind-quarter of the rat. 9% of $1\text{-}^{14}\text{C}$ palmitate taken up was oxidized to $^{14}\text{CO}_2$, account 7% of total oxygen consumption by the perfused tissue. Most label from $1\text{-}^{14}\text{C}$ -palmitate is found in lipid fraction of the muscle tissue. In spite of a 40% inhibition of palmitate oxidation, acetate only minor changes in the overall metabolism of palmitate. $\text{U-}^{14}\text{C}$ -acetate was mainly oxidized to $^{14}\text{CO}_2$, oxygen consumption due to oxidation of acetate accounted for 70-80% of the total oxygen uptake. Amounts of ^{14}C -acetate were found in muscle lipids. The addition of palmitate did not alter the metabolism of acetate. It is concluded that the presence of palmitate did not affect ^{14}C -acetate metabolism. The presence of acetate inhibited ^{14}C palmitate oxidation. The possible sites of interaction are discussed. The observed interaction will probably not contribute to any major extent to the disturbed lipid metabolism in animals and man during ethanol intake. No major changes in the tissue content of high-energy phosphate compounds were found in the presence of palmitate or acetate or both.

Key words: Acetate metabolism, palmitate metabolism, skeletal muscle, muscle perfusion, ethanol consumption

Acute and chronic administration of ethanol to man and animals is generally followed by metabolic alterations in a number of organs and tissues (Wallgren and Barry III 1970, and Mardones 1971, Kissin and Begleiter 1971). One characteristic alteration caused by ethanol is a disturbed lipid metabolism related to effects on adipose tissue or liver fat (Lieber 1974). Since the main metabolite of ethanol metabolism in the liver is acetate (Lundquist 1962), a short-chain fatty acid, it is of interest to study how this metabolite, in addition to a direct effect of ethanol, contributes to the disturbance of the lipid metabolism. We have shown that skeletal muscle is of quantitative importance for the elimination of acetate from the blood (Karlsson *et al.* 1975) and long-chain fatty acids are known to be important substrates for skeletal muscle (Keul *et al.* 1972). Thus, an altered combustion of long-

TABLE II The effect of acetate on the metabolic fate of $1\text{-}^{14}\text{C}$ -palmitate in the perfused hind-quarter of the rat

Exposure time (min)	Palmitate uptake ($\mu\text{mol/g wet weight}$)	Incorporation of label into		Oxygen consumption due to oxidation of ^{14}C -palmitate to $^{14}\text{CO}_2$ ($\mu\text{l/g}$)	Recovery of added label (%)
		$^{14}\text{CO}_2$	Water soluble metabolites in muscle tissue ($\mu\text{mol of palmitate/g wet weight}$)		
palmitate, 2 M	0.54 ± 0.06 (6)	0.087 ± 0.008 (6)	0.066 ± 0.010 (6)	7.3 ± 0.8 (6)	95 ± 1
palmitate, 2 M + acetate, 2 mM	0.50 ± 0.05 (5)	$0.062^b \pm 0.007$ (6)	$0.032^b \pm 0.003$ (6)	5.4 ± 0.8 (6)	93 ± 1

Values are expressed as mean \pm S.E. (n) the number of observations in parentheses. Experimental conditions are Table I and Methods.

Corrections have not been made for re-oxidation of NADH by lactate dehydrogenase.

Significantly different from perfusion without acetate, $p < 0.05$.

The oxygen consumption was not altered by the addition of acetate or palmitate or both (Table I). When the supply of exogenous substrates was restricted, as in the control experiment, the lactate concentration in the medium was essentially unchanged during the perfusion, but the addition of acetate or palmitate or both resulted in a net production of lactate (Table I).

Influence of acetate on $1\text{-}^{14}\text{C}$ -palmitate metabolism

About 9% of total ^{14}C -palmitate taken up was oxidized to CO_2 and about 7% was recovered in water-soluble metabolites in the muscle tissue (Table II). Most label taken up (about 90%) as found in the chloroform extract of the muscle tissue. The addition of acetate caused a statistically significant decrease in the incorporation of ^{14}C -palmitate into $^{14}\text{CO}_2$ and into water-soluble intermediates in the muscle tissue (Table II). In the presence of acetate about 60% of the label taken up was recovered in the chloroform extract of the muscle tissue.

On the basis of the oxygen consumption (Table I) and incorporation of ^{14}C -palmitate to $^{14}\text{CO}_2$ (Table II), it was calculated that palmitate oxidation accounted for about 7% of the oxidant metabolism of the perfused muscle (Table II). The addition of acetate did not significantly alter this figure.

Influence of palmitate on $1\text{-}^{14}\text{C}$ -acetate metabolism

About 40% of the total $1\text{-}^{14}\text{C}$ -acetate uptake was oxidized to CO_2 and H_2O as judged by the incorporation of label into $^{14}\text{CO}_2$ and about 3% was recovered in the chloroform extract of the muscle tissue (Table III). 46% of the acetate taken up was recovered as water-soluble compounds obtained in the perchloric acid extract of the muscle tissue. Acetate oxidation accounted for 20–30% of the total oxygen consumption. There was no significant change in acetate oxidation or incorporation of ^{14}C -acetate into lipids when palmitate was added (Table

TABLE 1 Rates of palmitate, acetate and oxygen uptake and net production of lactate in the perfused quarter of the rat

Additions to standard medium	Palmitate uptake	Acetate uptake ($\mu\text{mol/h g wet weight}$)	Oxygen consumption	Net production of lactate
Control ^a	—	—	27.3 ± 1.4 (5)	0.1 ± 0.1
Acetate 2 mM + infusion	—	8.0 ± 0.5 (7)	30.6 ± 1.8 (6)	0 ± 0
Palmitate 1 mM	0.94 ± 0.06 (8)	—	24.9 ± 2.0 (10)	2.0 ± 0.1
Palmitate, 1 mM } Acetate, 2 mM } + infusion	1.06 ± 0.07 (10)	7.0 ± 0.4 (8)	26.2 ± 1.1 (10)	3.0 ± 0.5

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Rat hind-limbs were perfused for 70 min. The standard medium contained glucose (3.3 mM). Substrates were added as min as a single dose (palmitate) or as a single dose followed by continuous infusion (acetate; 7.0 $\mu\text{mol/l}$) (Karlsson *et al.* 1975).

Data taken from Karlsson *et al.* (1976).

^a Significantly different from control $p < 0.05$.

As for b, $p < 0.01$.

At the end of the perfusion portion of the thigh muscle was rapidly frozen *in situ* with aluminium wire cooled in liquid nitrogen (Wollenberger *et al.* 1960). The frozen tissue (200–250 mg) was homogenized and treated with HClO_4 as previously described (Karlsson *et al.* 1975). The perchloric acid extract was analyzed for the content of adenosine-triphosphate (ATP) (Lamprecht and Trautwein 1962), adenosine-monophosphate (AMP) (Adam 1962), creatine-phosphate (CP) (Lamprecht and Stein 1964), inorganic phosphate (P_i) (Baginski *et al.* 1970) and lactate and pyruvate (Hofhorst *et al.* 1959).

Radioactivity measurement

The metabolic fates of the added substrates were studied with ^3H -acetate and ^3H -palmitate. The labelled substrates were added to give specific activity of 9 nCi/ μmol of acetate and 15 nCi/ μmol of palmitate in the medium. Added label was recovered at the end of the perfusion as described previously (Karlsson *et al.* 1975; Karlsson *et al.* 1976) except that radioactivity remaining in the perfusate at the end of the perfusion was measured by counting. Instead an aliquot of the plasma sample. Recovered radioactivity are given in Table II and III. Uptake of label from the medium was calculated from the difference in substrate concentration and the specific activity of the added substrate.

Statistical analysis

Student's *t*-test was used for statistical analysis, values of $p \leq 0.05$ being considered statistically significant.

Results

Rates of palmitate, acetate and oxygen uptake and net production of lactate

The addition of palmitate to the perfusion medium caused slight hemolysis of the red cells but this did not affect the arterial oxygen concentration.

Palmitate was added to give a final concentration of 1 mM in the medium and the uptake of palmitate was linear during the experimental period. Palmitate uptake was not altered by the addition of acetate (Table I). Acetate was continuously infused into the perfusion medium and the concentration of acetate (2 mM) was essentially constant throughout the experimental period. The uptake of acetate from the medium was not significantly altered when palmitate was included (Table I).

- 2.3.4. The effect of acetate and palmitate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hind-quarter of the rat at the end of the perfusion.

Conditions in perfused hind-quarter	Muscle tissue			Medium		
	Lactate ($\mu\text{mol/g wet}$)	Pyruvate ($\mu\text{mol/g}$)	L/P	Lactate (mM)	Pyruvate	L/P
control	1.13 ± 0.20 (6)	0.14 ± 0.02 (6)	7.6 ± 1.4 (6)	2.10 ± 0.14 (5)	0.11 ± 0.02 (5)	20.7 ± 4.4 (5)
acetate, 2 mM	$2.21^b \pm 0.47$ (4)	$0.08^b \pm 0.01$ (4)	$27.4^d \pm 8.6$ (4)	2.04 ± 0.13 (5)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)
palmitate, 1 mM	$3.43^d \pm 0.12$ (9)	0.12 ± 0.02 (9)	$39.7^d \pm 10.1$ (9)	$1.79^b \pm 0.14$ (10)	$0.16^b \pm 0.01$ (10)	$11.4^b \pm 0.8$ (10)
acetate, 1 mM + palmitate, 2 mM	$3.80^d \pm 0.41$ (10)	0.11 ± 0.01 (10)	$38.3^d \pm 5.6$ (10)	2.27 ± 0.25 (10)	$0.18^b \pm 0.01$ (10)	13.2 ± 1.1 (10)

Values are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions are the same as in Table I and methods.

Values taken from Karlsson *et al.* (1976).

^a significantly different from control perfusion, $p < 0.05$.

^b for $p < 0.01$.

^c for $p < 0.001$.

trofheldt *et al.* 1974). In addition, the metabolic fates of $1\text{-}^{14}\text{C}$ palmitate (Table II) (Beatty and Bock 1971) and $1\text{-}^{14}\text{C}$ oleate (Reimer *et al.* 1975) were essentially the same *i.e.* most ^{14}C -label was found in muscle lipids, while about 10% of the ^{14}C -fatty acid taken up was oxidized to $^{14}\text{CO}_2$. These similarities between palmitate and oleate metabolism have also been found in man (Hagenfeldt 1975).

In contrast to palmitate, most ^{14}C -acetate was oxidized to $^{14}\text{CO}_2$ and H_2O while only minor amounts of ^{14}C -label were found in lipids (Table III). If the incorporation of label into $^{14}\text{CO}_2$ from ^{14}C -palmitate is expressed as μmol of acetate the figure in Table II will be about 0.6 (0.08 S.E.) μmol of acetate/g. Thus, there is a 5 times higher incorporation of radioactivity into $^{14}\text{CO}_2$ from ^{14}C -acetate, than from ^{14}C -palmitate. Since most palmitate is stored as lipids, while acetate was mainly oxidized, the two compounds enter different metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids is compared to long-chain fatty acids, has previously been observed in skeletal muscle (Fritz 1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while the activation of long-chain fatty acids is mainly extra-mitochondrial (Brenner 1970, Aas 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1962), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathways *i.e.* the citric acid cycle. Palmitate, on the other hand, will pass through several regulatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β -oxidation steps) before it enters the citric acid cycle, where $^{14}\text{CO}_2$ is formed. In agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U- 14 C-acetate in the perfused hind-quarter of the rat.

Additions to standard medium	Acetate uptake ($\mu\text{mol/h g wet weight}$)	Incorporation of label into		Oxygen consumption due to oxidation of ^{14}C -acetate to $^{14}\text{CO}_2$ (μl) ^b	Ratio of label (s)
		$^{14}\text{CO}_2$ ($\mu\text{mol of acetate/h g wet weight}$)	Lipids ($\mu\text{mol of acetate/h g wet weight}$)		
^{14}C -acetate, 2 mM					
+ infusion	8.3 ± 0.4 (6)	3.17 ± 0.12 (6)	0.13 ± 0.04 (6)	30.6 ± 1.7 (6)	91
^{14}C -acetate, 2 mM					
+ infusion	7.9 ± 0.3 (6)	3.65 ± 0.34 (6)	0.13 ± 0.02 (6)	28.5 ± 4.2 (6)	93
Palmitate 1 mM					

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

Data taken from Karlsson *et al.* (1976).

^b Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

III) but the content of ^{14}C label in the perchloric acid extract was increased to about ($p < 0.05$).

Lactate and pyruvate concentrations and L/P ratios in the muscle tissue and the medium at the end of the perfusion

The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palmitate, acetate or both were added to the medium (Table IV). In the presence of palmitate changes were mainly due to an increased lactate concentration, since the pyruvate concentration was essentially unchanged (Table IV). However, with acetate as the only substrate the change in L/P ratio in the muscle tissue was due to an increase in lactate and a decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tissue were not reflected by similar changes in the medium.

Tissue content of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy charge" in the muscle tissue

The tissue content of ATP, ADP, AMP, CP and P_i was not significantly altered by the addition of acetate, as compared to the control (Table V). However, in the presence of palmitate, acetate caused a statistically significant decrease in the tissue content of ATP and P_i , as compared to the perfusion with palmitate but no acetate (Table V). The decrease in high-energy phosphate compounds and P_i by acetate in the presence of palmitate was not reflected by changes in the "phosphate potential" ($[\text{ATP}]/[\text{ADP}] + [\text{P}_i]$) (Atkinson, 1968) or the ratio $[\text{ADP}]/[\text{ATP}] + [\text{AMP}]$ which represents the mass action ratio of the adenine kinase reaction (Table V).

Discussion

Palmitate uptake in the present study (Table I) was of the same order of magnitude as the uptake of oleate in the perfused rat hind-quarter reported by others (Reimer *et al.*, 1975).

IV The effect of acrylate and palmitate or both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hind-quarter of the rat at the end of the perfusion.

dose to and medium	Muscle tissue			Medium		
	Lactate ($\mu\text{mol/g}$)	Pyruvate ($\mu\text{mol/g}$)	L/P	Lactate (mM)	Pyruvate (mM)	L/P
rest	1.15 (6)	0.14 \pm 0.02 (6)	7.6 \pm 1.4 (6)	2.18 \pm 0.14 (5)	0.11 \pm 0.02 (5)	20.7 \pm 4.4 (5)
ins. 2 mM^a 5 min	2.21 \pm 0.47 (4)	0.08 \pm 0.01 (4)	27.4 \pm 8.6 (4)	2.04 \pm 0.13 (6)	0.13 \pm 0.01 (6)	16.2 \pm 1.4 (6)
ins. 1 mM	3.45 \pm 0.12 (7)	0.12 \pm 0.02 (7)	29.7 \pm 10.1 (7)	1.79 \pm 0.14 (10)	0.16 \pm 0.01 (10)	11.4 \pm 0.8 (10)
ins. 1 mM ins. 10 mM 5 min	3.80 \pm 0.41 (10)	0.11 \pm 0.01 (10)	35.3 \pm 5.6 (10)	2.27 \pm 0.25 (10)	0.18 \pm 0.01 (10)	13.2 \pm 1.1 (10)

its are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions, see Table I and Methods.

^a as taken from Karlsson *et al.* (1974).

significantly different from control perfusion, $p < 0.05$.

for $n, p < 0.01$

for $n, p < 0.001$

Heldt *et al.* 1974). In addition, the metabolic fates of 1-¹⁴C-palmitate (Table II) (Beatty & Bock 1971) and 1-¹⁴C-oleate (Rehner *et al.* 1975) were essentially the same (*i.e.* most label was found in muscle lipids, while about 10% of the ¹⁴C-fatty acid taken up was fixed to ¹⁴CO₂). These similarities between palmitate and oleate metabolism have also been found in man (Hagenfeldt 1975).

In contrast to palmitate, most ¹⁴C-acetate was oxidized to ¹⁴CO₂ and H₂O while only small amounts of ¹⁴C-label were found in lipids (Table III). If the incorporation of label to ¹⁴CO₂ from ¹⁴C-palmitate is expressed as μmol of acetate, the figure in Table II will be about 0.6 (0.08 S.E.) μmol of acetate/h g. Thus, there is a 5 times higher incorporation of radioactivity into ¹⁴CO from ¹⁴C-acetate, than from ¹⁴C-palmitate. Since most palmitate is stored in lipids, while acetate was mainly oxidized, the two compounds enter different metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids compared to long-chain fatty acids, has previously been observed in skeletal muscle (Harris 1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while the activation of long-chain fatty acids is mainly extra-mitochondrial (Bresler 1970, Aas 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1962), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathways (*i.e.* the citric acid cycle). Palmitate, on the other hand, will pass through several preliminary steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β -oxidation steps) before it enters the citric acid cycle, where ¹⁴CO₂ is formed. In agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U-¹⁴C-acetate in the perfused hind-quarter of the rat.

Additions to standard medium	Acetate uptake ($\mu\text{mol/h g wet weight}$)	Incorporation of label into		Oxygen consumption due to oxidation of ¹⁴ C acetate to ¹⁴ CO ₂ (%) ^b	R _{ox} of label
		¹⁴ CO ₂ ($\mu\text{mol of acetate/h g wet weight}$)	Lipids ($\mu\text{mol of acetate/h g wet weight}$)		
¹⁴ C-acetate 2 mM + infusion	8.3 \pm 0.4 (6)	3.17 \pm 0.22 (6)	0.12 \pm 0.04 (6)	20.6 \pm 1.7 (6)	11
¹⁴ C-acetate, 2 mM + infusion	7.9 \pm 0.3 (6)	3.65 \pm 0.34 (6)	0.13 \pm 0.02 (6)	22.5 \pm 4.2 (4)	9
Palmitate, 2 mM					

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experiments see Table I and Methods.

^a Data taken from Karlsson *et al.* (1976)

^b Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

III) but the content of ¹⁴C label in the perchloric acid extract was increased to about 50% ($p < 0.05$).

Lactate and pyruvate concentrations and L/P ratios in the muscle tissue and the medium at the end of the perfusion

The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palmitate or both were added to the medium (Table IV). In the presence of palmitate changes were mainly due to an increased lactate concentration, since the pyruvate concentration was essentially unchanged (Table IV). However, with acetate as the only substrate the change in L/P ratio in the muscle tissue was due to an increase in lactate and a decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tissue were not reflected by similar changes in the medium.

Tissue content of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy charge" in the muscle tissue

The tissue content of ATP, ADP, AMP, CP and P_i was not significantly altered by addition of acetate as compared to the control (Table V). However, in the presence of palmitate acetate caused a statistically significant decrease in the tissue content of ATP and P_i as compared to the perfusion with palmitate but no acetate (Table V). The changes in high-energy phosphate compounds and P_i by acetate in the presence of palmitate were not reflected by changes in the "phosphate potential" ([ATP]/[ADP] [P_i]) (Atkinson) or the ratio [ADP]/[ATP] [AMP] which represents the mass action ratio of the adenine kinase reaction (Table V).

Discussion

Palmitate uptake in the present study (Table I) was of the same order of magnitude as the uptake of oleate in the perfused rat hind-quarter reported by others (Reimer *et al.*

- IV The effect of acetate and palmitate on both on the concentrations of lactate and pyruvate and the [lactate]/[pyruvate] (L/P) ratio in the medium and the muscle tissue of the perfused hind-quarter of the rat at the end of the perfusion.

values to ratified medium	Muscle tissue			Medium		
	Lactate ($\mu\text{mol/g wet weight}$)	Pyruvate	L/P	Lactate (mM)	Pyruvate	L/P
control	1.15 ± 0.20 (6)	0.14 ± 0.02 (6)	7.6 ± 1.4 (6)	2.18 ± 0.14 (7)	0.11 ± 0.01 (5)	20.7 ± 4.4 (5)
acet. 2 mM	$2.21^b \pm 0.47$ (4)	$0.08^b \pm 0.01$ (6)	$27.4^b \pm 9.6$ (4)	2.04 ± 0.13 (6)	0.13 ± 0.01 (6)	16.2 ± 1.4 (6)
acetate 1 mM	$3.45^c \pm 0.12$ (9)	0.12 ± 0.03 (9)	$39.7^c \pm 10.1$ (9)	$1.79^b \pm 0.14$ (10)	$0.16^b \pm 0.01$ (10)	$11.4^b \pm 0.8$ (10)
acetate 1 mM + acet. 2 mM palmitate	$3.80^b \pm 0.41$ (10)	0.11 ± 0.01 (10)	$38.3^b \pm 5.6$ (10)	2.27 ± 0.15 (10)	$0.18^b \pm 0.01$ (10)	13.2 ± 1.1 (10)

Values are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions as in Table I and Methods.

Data taken from Karlsson *et al.* (1976).

Significantly different from control perfusion, $p < 0.05$.

^b vs. control, $p < 0.01$.

^c vs. control, $p < 0.001$.

rotfeldt *et al.* 1974). In addition, the metabolic fates of 1-¹⁴C-palmitate (Table II) (Beatty and Bock 1971) and 1-¹⁴C-oleate (Reimer *et al.* 1975) were essentially the same: most ¹⁴C-label was found in muscle lipids, while about 10% of the ¹⁴C-fatty acid taken up was oxidized to ¹⁴CO₂. These similarities between palmitate and oleate metabolism have also been found in man (Hagenfeldt 1975).

In contrast to palmitate, most ¹⁴C-acetate was oxidized to ¹⁴CO and H₂O while only minor amounts of ¹⁴C-label were found in lipids (Table III). If the incorporation of label into ¹⁴CO₂ from ¹⁴C-palmitate is expressed as μmol of acetate the figure in Table II will be about 0.6 (0.08 μmol of acetate/h g. Thus, there is a 3 times higher incorporation of radioactivity into ¹⁴CO₂ from ¹⁴C-acetate, than from ¹⁴C-palmitate. Since most palmitate is stored as lipid, while acetate was mainly oxidized, the two compounds enter different metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids compared to long-chain fatty acids, has previously been observed in skeletal muscle (Frost 1961) and in intact rats *in vivo* (Krechner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while the activation of long-chain fatty acids is mainly extra-mitochondrial (Bresler 1970, Aus 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1962), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathways—the citric acid cycle. Palmitate, on the other hand, will pass through several regulatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β -oxidation steps) before it enters the citric acid cycle, where ¹⁴CO₂ is formed. In agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle, but is mainly incorporated into lipids before

TABLE III The effect of palmitate on the metabolic fate of U- 14 C-acetate in the perfused hind-quarter of the rat.

Additions to standard medium	Acetate uptake (μ mol/h g wet weight)	Incorporation of label into		Oxygen consumption due to oxidation of 14 C-acetate to 14 CO $_2$ (μ mol/h g wet weight)	Loss of label (%)
		14 CO $_2$ (μ mol of acetate/h g wet weight)	Lipids (μ mol of acetate/h g wet weight)		
14 C-acetate, 2 mM ^a					
+ Infusion	8.3 ± 0.4 (6)	3.17 ± 0.22 (6)	0.12 ± 0.04 (6)	20.6 ± 1.7 (6)	91.4
14 C-acetate, 2 mM					
+ Infusion	7.9 ± 0.3 (6)	3.65 ± 0.34 (6)	0.13 ± 0.02 (6)	28.5 ± 4.2 (6)	91.1
Palmitate 1 mM					

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions see Table I and Methods.

^a Data taken from Karlsson *et al.* (1976).

^b Correction has not been made for re-oxidation of NADH by lactate dehydrogenase.

III) but the content of 14 C-label in the perchloric acid extract was increased to ab ($p < 0.05$).

Lactate and pyruvate concentrations and L/P ratios in the muscle tissue and the medium at the end of the perfusion

The [lactate]/[pyruvate] (L/P) ratio in the muscle tissue was elevated when palmitate or both were added to the medium (Table IV). In the presence of palmitate changes were mainly due to an increased lactate concentration, since the pyruvate concentration was essentially unchanged (Table IV). However, with acetate as the only substrate the change in L/P ratio in the muscle tissue was due to an increase in lactate and a decrease in the pyruvate concentration. The changes in L/P ratios in the muscle tissue were not reflected by similar changes in the medium.

Tissue content of adenine-nucleotides, creatine-phosphate and inorganic phosphate and the "energy charge" in the muscle tissue

The tissue content of ATP, ADP, AMP, CP and P_i was not significantly altered by addition of acetate as compared to the control (Table V). However, in the presence of palmitate acetate caused a statistically significant decrease in the tissue content of ATP and P_i , as compared to the perfusion with palmitate but no acetate (Table V). The changes in high-energy phosphate compounds and P_i by acetate in the presence of palmitate were not reflected by changes in the "phosphate potential" ($[ATP]/[ADP][P_i]$) (Atkins) or the ratio $[ADP]/[ATP][AMP]$ which represents the mass action ratio of the adenosine kinase reaction (Table V).

Discussion

Palmitate uptake in the present study (Table I) was of the same order of magnitude as uptake of oleate, in the perfused rat hind-quarter reported by others (Reimer *et al.*

IV The effect of acetate and palmitate on both on the concentrations of lactate and pyruvate and the lactate/pyruvate (L/P) ratio in the medium and the muscle tissue of the perfused hind-quarter of the rat at the end of the perfusion

ratios to control medium	Muscle tissue			Medium		
	Lactate ($\mu\text{mol/g}$ wet weight)	Pyruvate ($\mu\text{mol/g}$ wet weight)	L/P	Lactate (mM)	Pyruvate (mM)	L/P
n^a	1.13 \pm 0.20 (6)	0.14 \pm 0.02 (6)	7.6 \pm 1.4 (6)	2.18 \pm 0.14 (5)	0.11 \pm 0.03 (5)	20.7 \pm 4.4 (5)
ac. 2 mM ^b	2.31 ^a \pm 0.47 (4)	0.08 ^b \pm 0.01 (6)	27.4 ^a \pm 8.6 (4)	2.04 \pm 0.13 (6)	0.13 \pm 0.01 (6)	16.3 \pm 1.4 (6)
pal. 1 mM	3.45 ^a \pm 0.12 (7)	0.12 \pm 0.02 (7)	29.7 ^a \pm 10.1 (7)	1.79 ^b \pm 0.14 (10)	0.16 ^b \pm 0.01 (10)	11.2 ^a \pm 0.9 (10)
pal. 1 mM n. mM muscle	3.80 ^a \pm 0.41 (10)	0.11 \pm 0.01 (10)	38.3 ^a \pm 5.6 (10)	2.27 \pm 0.25 (10)	0.18 ^a \pm 0.01 (10)	13.3 \pm 1.1 (10)

n are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions Table I and Methods.

^a taken from Karlsson *et al.* (1976).

^b statistically different from control perfusion, $p < 0.05$.

or $n, p < 0.01$

for $n, p < 0.001$

(Feldt *et al.* 1974). In addition, the metabolic fates of 1-¹⁴C-palmitate (Table II) (Beatty Boock 1971) and 1-¹⁴C-oleate (Reimer *et al.* 1975) were essentially the same, i.e. most label was found in muscle lipids, while about 10% of the ¹⁴C-fatty acid taken up was used to ¹⁴CO₂. These similarities between palmitate and oleate metabolism have also been found in man (Hagenfeldt 1975).

In contrast to palmitate most ¹⁴C-acetate was oxidized to ¹⁴CO₂ and H₂O while only small amounts of ¹⁴C-label were found in lipids (Table III). If the incorporation of label ¹⁴CO₂ from ¹⁴C-palmitate is expressed as μmol of acetate, the figure in Table II will become 0.6 (0.08 S.E.) μmol of acetate/h g. Thus, there is a 5 times higher incorporation of activity into ¹⁴CO₂ from ¹⁴C-acetate, than from ¹⁴C-palmitate. Since most palmitate is stored as lipids, while acetate was mainly oxidized, the two compounds enter different metabolic pathways in the muscle cell at rest. A faster oxidation of short-chain fatty acids compared to long-chain fatty acids, has previously been observed in skeletal muscle (1961) and in intact rats *in vivo* (Kirschner and Harris 1961). It is known that the activation of short-chain fatty acids in skeletal muscle is localized within the mitochondrion while activation of long-chain fatty acids is mainly extra-mitochondrial (Brenner 1970, Aas 1971). Since acetate easily penetrates the mitochondrial membrane (Spencer and Lowenstein 1971), this compound can, after activation to acetyl-CoA, immediately enter the oxidative pathway, i.e. the citric acid cycle. Palmitate, on the other hand, will pass through several obligatory steps (activation, conversion to carnitine derivative, re-activation to acyl-CoA and finally β -oxidation steps) before it enters the citric acid cycle, where ¹⁴CO₂ is formed. In agreement with our results, it has been found previously that palmitate is not used as an immediate energy source in the resting muscle, but is mainly incorporated into lipids before

TABLE V The effect of acetate and palmitate or both on the concentrations of adenosine-nucleotides, inorganic phosphate and inorganic phosphate and the energy-charge of the resting, perfused hind-quarter at the end of the perfusion.

Additions to standard medium	ATP (μ mol/g wet weight)	ADP	AMP	CP	PI	ATP ADP PI	ADP ATP-PI ^a
Control ^a	5.01 \pm 0.31 (6)	0.70 \pm 0.05 (6)	0.024 \pm 0.006 (6)	16.4 \pm 1.5 (6)	16.0 \pm 1.2 (6)	0.46 \pm 0.03 (6)	5.5 \pm 1.5 (6)
Acetate, 2 mM ^a + infusion	5.33 \pm 0.16 (6)	0.77 \pm 0.09 (6)	0.026 \pm 0.004 (5)	16.0 \pm 1.6 (5)	15.1 \pm 1.6 (6)	0.51 \pm 0.07 (6)	5.3 \pm 1.6 (5)
Palmitate, 1 mM	5.93 ^b \pm 0.16 (8)	0.77 \pm 0.04 (8)	0.024 \pm 0.004 (6)	17.3 \pm 1.5 (8)	14.1 \pm 1.3 (8)	0.99 \pm 0.09 (8)	5.6 \pm 1.9 (8)
Palmitate, 1 mM Acetate, 2 mM + infusion	5.03 ^c \pm 0.6 (10)	0.67 ^d \pm 0.04 (10)	0.019 \pm 0.003 (3)	15.2 \pm 1.3 (10)	10.5 ^e \pm 0.6 (9)	0.69 \pm 0.03 (9)	5.3 \pm 1.2 (5)

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions, see Table I and Methods.

^a Data taken from Karlsson *et al.* (1976).

^b Significantly different from control perfusion, $p < 0.05$.

^c As for ^a, $p < 0.01$.

^d As for ^a, $p < 0.001$.

^e Significantly different from perfusion with palmitate but without acetate, $p < 0.05$.

^f As for ^d, $p < 0.01$.

it is oxidized (Issekutz 1970, Havel 1970). This might explain the finding that, in spite of excessive exogenous substrates, the oxidation of palmitate and acetate together accounts for only 35% of the total oxygen consumption in the perfused tissue (Table II) and Reimer *et al.* (1975) found that most label from 1-¹⁴C-oleate incorporated into lipids in perfused rat hind-quarter was found in the triglyceride fraction.

The addition of acetate to the medium caused a 40% decrease in the incorporation of label from ¹⁴C-palmitate into ¹⁴CO₂ and into water-soluble intermediates in the muscle tissue (Table II). The concomitant tendency of an increase in the amount of label in lipid extract of the muscle tissue in the presence of acetate indicates that the ¹⁴C-palmitate was diverged to lipid synthesis. Although this effect was not statistically significant, it may explain the decreased incorporation of ¹⁴C-palmitate into ¹⁴CO₂, since the inhibition only affects the distribution of palmitate between the oxidative and synthetic reactions, about 3%. If the incorporation of label into ¹⁴CO₂ is expressed as per cent of palmitate uptake (Table II). Furthermore, we did not find any evidence of competition between palmitate and acetate at the same enzyme site, since changes in ¹⁴C-acetate metabolism were not affected when palmitate was added and the simultaneous presence of the two substrates did not affect the uptake of either of them. Thus, at present we are not able to stipulate the exact site of interaction of acetate on ¹⁴C-palmitate oxidation, but one possible explanation may be a competition between the two substrates for CoA. There are no published studies on the interaction between short-chain and long-chain fatty acids in skeletal muscle at rest, and previous studies on rat heart have given conflicting results. Shipp (1964) found that in the perfused rat heart acetate did not affect palmitate uptake but inhibited palmitate oxidation, thus confirming our findings in skeletal muscle. On the other hand, Bethencourt

Lee (1966) found no effects of acetate on ^{14}C -palmitate metabolism. In rat heart mitochondria Lee and Vabourey (1974) found that acetate did not affect palmitate oxidation, but palmitate depressed acetate oxidation and they suggested that the pathways for oxidation of the substrates in heart mitochondria are compartmentalized. Similar results from studies on rat liver mitochondria have been reported by Cederbaum and Rubin (1975).

It is reported recently (Karlsson *et al.* 1976) the addition of exogenous substrates to the perfusion medium will cause a net production of lactate by the perfused hind-quarter (Table I).

This effect is discussed in the cited paper (*op. cit.*). In a previous study we found no significant effect on the lactate production when acetate was continuously infused into the perfusion medium (Karlsson *et al.* 1975). An explanation to this discrepancy might be the different initial concentration of lactate in the perfusion medium in the two studies. Dunn and Critz (1975) found that in the dog hind limb the arterio-venous difference of lactate is a function of the lactate concentration in the arterial blood. Thus, at concentrations below about 1 mM release or uptake of lactate occurred. In the present study the initial lactate concentration in the control experiment without insulin was 1.9 mM and in the previous study 1 mM (Karlsson *et al.* 1975). Obviously a balance between release and uptake of lactate is established at about 2 mM, when the supply of exogenous substrates is restricted. Renner *et al.* (1975), in their studies on glucose and fatty acid metabolism in the perfused hind-quarter found no effect of oleate on the lactate production in the absence of insulin. The increase in lactate production (Table IV), caused by the addition of acetate or palmitate or both, was followed by an increase in the tissue concentration of lactate and a corresponding elevation of the L/P ratio in the tissue (Table V). These changes were not reflected by similar changes in the medium. Renner *et al.* (*op. cit.*) also found, with the same experimental technique, that the addition of oleate caused no changes in the L/P ratio in the perfusion medium.

In conclusion, acetate affected palmitate metabolism only slightly if the effect is related to the total palmitate uptake by the muscle. However there was 40% inhibition of palmitate oxidation, which indicates a profound effect of acetate on palmitate oxidation to CO_2 . At present the site of this inhibition is not known. Extrapolation of our findings to the situation *in vivo* indicates that acetate will not contribute to an altered lipid metabolism in other tissues through its action on skeletal muscle. However the present results will probably not reflect the situation in the stimulated skeletal muscle, since in this situation the energy requirements will increase and the importance of long-chain fatty acids for the oxidative metabolism is enhanced (Keul *et al.* 1972). Thus, any inhibition of the oxidative metabolism of long-chain fatty acids may be of great significance during exercise.

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TABLE V The effect of acetate and palmitate or both on the concentrations of adenine-nucleotides, on phosphate and inorganic phosphate and the "energy-charge" of the resting, perfused hind-limb at the end of the perfusion.

Additions to standard medium	ATP ($\mu\text{mol/g}$ wet weight)	ADP ($\mu\text{mol/g}$ wet weight)	AMP	CP	PI	$\frac{\text{ATP}}{\text{ADP} + \text{PI}}$	$\frac{\text{ADP}}{\text{ATP} + \text{PI}}$
Control ^a	5.01 ± 0.31 (6)	0.70 ± 0.03 (6)	0.024 ± 0.006 (6)	16.4 ± 1.5 (6)	16.0 ± 1.2 (6)	0.46 ± 0.03 (6)	0.53 ± 0.03 (6)
Acetate, 2 mM + infusion	5.33 ± 0.16 (6)	0.77 ± 0.09 (6)	0.026 ± 0.004 (5)	16.0 ± 1.6 (5)	15.1 ± 1.6 (6)	0.51 ± 0.07 (6)	0.49 ± 0.07 (5)
Palmitate, 1 mM	$5.93^b \pm 0.16$ (8)	0.77 ± 0.04 (8)	0.024 ± 0.004 (6)	17.3 ± 1.5 (8)	14.1 ± 1.3 (8)	0.59 ± 0.09 (8)	0.41 ± 0.09 (6)
Palmitate, 1 mM + Acetate 2 mM + infusion	$5.03^c \pm 0.16$ (10)	$0.67^d \pm 0.04$ (10)	0.019 ± 0.003 (5)	15.2 ± 1.2 (10)	$10.5^{c,d} \pm 0.6$ (9)	0.69 ± 0.03 (9)	0.31 ± 0.03 (5)

Results are expressed as mean \pm S.E. with the number of observations in parentheses. Experimental conditions: see Table I and Methods.

Data taken from Karlsson *et al.* (1976).

^a Significantly different from control perfusion, $p < 0.05$.

^b As for ^a, $p < 0.01$.

^c As for ^a, $p < 0.001$.

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^e As for ^d, $p < 0.01$.

It is oxidized (Isakutz 1970; Havel 1970). This might explain the finding that, in spite of excessive exogenous substrates, the oxidation of palmitate and acetate together accounted for only 35% of the total oxygen consumption in the perfused tissue (Table II) and Reimer *et al.* (1975) found that most label from 1-¹⁴C-oleate incorporated into lipids in the perfused rat hind-quarter was found in the triglyceride fraction.

The addition of acetate to the medium caused a 40% decrease in the incorporation of label from ¹⁴C-palmitate into ¹⁴CO₂ and into water-soluble intermediates in the muscle tissue (Table II). The concomitant tendency of an increase in the amount of label in the lipid extract of the muscle tissue in the presence of acetate indicates that the ¹⁴C-palmitate was diverged to lipid synthesis. Although this effect was not statistically significant, it may explain the decreased incorporation of ¹⁴C-palmitate into ¹⁴CO₂, since the inhibition only affects the distribution of palmitate between the oxidative and synthetic reaction pathways (about 3%, if the incorporation of label into ¹⁴CO₂ is expressed as per cent of palmitate uptake (Table II)). Furthermore, we did not find any evidence of competition between palmitate and acetate at the same enzyme site, since changes in ¹⁴C-acetate metabolism were not seen when palmitate was added and the simultaneous presence of the two substrates did not affect the uptake of either of them. Thus, at present we are not able to stipulate the exact site of interaction of acetate on 1-¹⁴C-palmitate oxidation, but one possible explanation may be a competition between the two substrates for CoA. There are no published studies on the interaction between short-chain and long-chain fatty acids in skeletal muscle at rest, and previous studies on rat heart have given conflicting results. Shipp (1964) found that in the perfused rat heart acetate did not affect palmitate uptake but inhibited palmitate oxidation, thus confirming our findings in skeletal muscle. On the other hand, Bethencourt

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TABLE V The effect of acetate and palmitate or both on the concentrations of adenine-nucleoside phosphate and inorganic phosphate and the energy-charge of the resting, perfused rat hind-quarter at the end of the perfusion

Additions to standard medium	ATP ($\mu\text{mol/g wet weight}$)	ADP	AMP	CP	Pi	ATP ADP + P _i	A
Control ^a	5.01 \pm 0.31 (6)	0.70 \pm 0.05 (6)	0.024 \pm 0.006 (6)	16.4 \pm 1.5 (6)	16.0 \pm 1.2 (6)	0.46 \pm 0.03 (6)	5
Acetate, 2 mM + infusion	5.33 \pm 0.16 (6)	0.77 \pm 0.09 (6)	0.026 \pm 0.004 (5)	16.0 \pm 1.6 (5)	15.1 \pm 1.6 (6)	0.51 \pm 0.07 (6)	5
Palmitate, 1 mM	5.93 ^b \pm 0.16 (8)	0.77 \pm 0.04 (8)	0.024 \pm 0.004 (6)	17.3 \pm 1.5 (8)	14.1 \pm 1.3 (8)	0.59 \pm 0.09 (8)	5
Palmitate 1 mM Acetate, 2 mM + infusion	5.03 ^f \pm 0.26 (10)	0.67 ^g \pm 0.04 (10)	0.019 \pm 0.003 (5)	15.2 \pm 1.2 (10)	10.5 ^h \pm 0.6 (9)	0.69 \pm 0.03 (9)	5

Results are expressed as mean \pm S.E. with the number of observations in parentheses. E peristalsis: see Table I and Methods.

Data taken from Karlsson *et al.* (1976).

^b Significantly different from control perfusion, $p < 0.05$.

^c As for ^a, $p < 0.01$.

^d As for ^a, $p < 0.001$.

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Contractile Responses to Noradrenaline. Varying Dependence on External Calcium of Consecutive Vascular Segments of Perfused Rat Hindquarters

By

MORLEY C. SUTTER, MARGARETA HALLBÄCK, JOHN V. JONES and BJÖRN FOLKOW

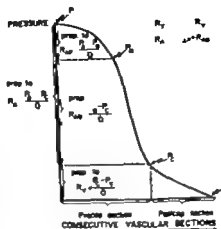
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Abstract

SUTTER, M. C., M. HALLBÄCK, J. V. JONES and B. FOLKOW. Contractile responses to noradrenaline. Varying dependence on external calcium of consecutive vascular segments of perfused rat hindquarters. *Acta physiol. scand.* 1977 99 166-172.

To study the dependency of vascular smooth muscle contractions, produced by noradrenaline, on concentration of extracellular calcium, experiments were performed on adult male Wistar rats. The hemodynamic characteristics of the isolated, perfused hindquarters were investigated from arterial dilatation up to maximal, noradrenaline-induced constriction under constant flow conditions. Pressure responses to noradrenaline in the consecutive segments of the vascular bed ("proximal" and "distal" precapillary resistance vessels and postcapillary resistance vessels) were determined at low (10^{-8} M) and at normal ($1.5-2.0$ mM) Ca^{++} concentrations in the perfusate. Dependence on external calcium was greater peripherally. The smallest pre- and postcapillary resistance vessels are the most dependent on the larger "proximal" precapillary resistance vessels are the least dependent on the availability of extracellular calcium. The results illustrate the considerable differentiation of smooth muscle, depending on its location along the vascular circuit. They further indicate that it is likely that the hemodynamically important microvessels are highly dependent on extrinsic Ca^{++} sources not only concerning their myogenic tone, but also in their maintenance of normally pronounced "myogenic tone".

It is known that *in vitro* various types of smooth muscle in larger vessels differ considerably in several important respects, including their dependence on external calcium (Ca^{++}) for contraction to noradrenaline (NA). At one extreme is the aorta which continues to contract in response to NA in the absence of external Ca^{++} and at the other is the portal-mesenteric vein which will not contract without external Ca^{++} . This raises the question as to which is the most appropriate analogue of the pre- and postcapillary microvessels responsible for the important resistance and capacitance adjustments which in the end determine the hemodynamic characteristics.



1 Schematic representation of the resistance to flow in a vascular circuit illustrating the pressure P across the consecutive resistance sections. The P at each pressure were measured or calculated the perfused rat hindquarter are indicated by P_1 , P_2 and P_3 respectively (see text)

There is, however, a paucity of information about requirements for extracellular Ca^{++} in vascular smooth muscle contractions produced by NA *in situ*. Haddy (1960) studied the effects of increased Ca^{++} on responses to NA of the blood vessels of the perfused dog forelimb. Jones *et al.* (1973) and Hinkle (1966) have reported the effects of reduced Ca^{++} on responses to NA in rat mesenteric vascular preparations and tail artery respectively. However, both of these preparations may be more representative in several respects of conduit vessels than of the true resistance vessels lying adjacent to the capillaries. As it seems important to gain more information about the functional characteristics of these important microvessels, experiments were performed to examine the effects of very low external Ca^{++} concentrations on the respective dose response curves to NA in the consecutive vascular segments of perfused rat hind quarters. The results indicate that the dependence on external Ca^{++} increases considerably as one proceeds peripherally in the vascular tree with the smallest pre- and postcapillary vessels being most and larger arteries least dependent.

A brief report based on some of these results has been presented elsewhere (Folkow *et al.* 1976).

Methods

The results are compiled from perfusion of the hindquarters of 26 male Wistar rats with an artificial plasma substitute as described previously (Folkow *et al.* 1970, 1974) but utilizing Dextran, 4%, instead of Ficoll as colloid. In each preparation the rise in arterial inflow pressure (P) in response to a given NA infusion reflects the changes in total resistance to flow (R_T) of the hindquarter vascular bed and as measured in all animals. To differentiate between the responses of the larger more proximal and smaller more distal precapillary vessels, pressure (P) was also measured in a small limb artery (external diameter at maximum distension 200–300 μm) in 6 animals. Under constant flow conditions P then gives a measure of the "proximal" precapillary resistance (R_{AP}) and its relationship to the total resistance to flow (R_T). In 9 of the 16 rats an isogravimetric technique (Folkow *et al.* 1974) was used to differentiate between responses of pre- and postcapillary resistance vessels, by measuring arterial inflow pressure (P_A), venous outflow pressure (P_V) and tissue volume. Mean capillary pressure (P_C), and hence pre- and postcapillary resistances (R and R_P , respectively) could then be calculated by quantitative evaluations of the changes in capillary filtration produced by mechanically and transiently altering inflow and outflow pressures,

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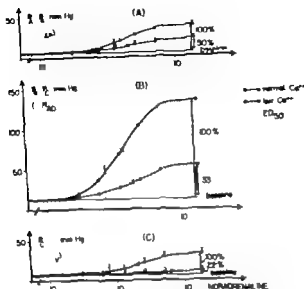
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1. Legends as for Fig. 2, except: A) P is pressure measured in a small artery with an external radius capsule calcitonin (1 cm exposed to locally applied papaverine) around $0.1-0.15$ (see text). $P_A - P$ is proportional to R_{AP} ("proximal" precapillary resistance) since flow was kept constant throughout its total vertical bath represent $M \pm S.E.$ from 6 animals. B) $P - P$ is proportional to R_{AD} ("distal" capillary resistance). The points are values calculated from the curves shown in Fig. 2, 3A and 3C. C) $P - P$ is proportional to postcapillary vascular resistance. The points represent $M \pm S.E.$ from 8 or 9 min. As in Fig. 2 the vertical dashed line in A, B and C indicates noradrenaline concentration of 10^{-6} M the horizontal dashed line depressed by crosses indicates how much the noradrenaline concentration must be increased at low calcium to elicit the same pressure response.

infusion coincide closely with observations in the intact rat as to the relative magnitude the resistance increases in the "proximal" resistance vessels. They imply that the normal two control of resistance is not only localized to the distal microvessels but involves a relatively extensive portion of the precapillary vessels, including their larger proximal ones as well. There is also clear tendency for functional differentiation among the proximal and distal vessels in the rat. The proximal resistance vessels evidently contribute relatively little to the centrally directed vasoconstrictor fibre control than to the locally originated "myogenic" resistance, which consequently resides mainly in the distal precapillary resistance vessels under basal conditions. It is also though, of course, with a considerable spatial overlap.

Concerning the situation during *in vitro* perfusion the concentration of Ca^{++} in the venous effluent was from 0.4 to 0.5 mM during perfusion with low Ca^{++} solutions (0.2 mM). The pressure responses to increasing concentrations of NA in consecutive segments of the vascular bed, perfused with normal or low Ca^{++} solutions, are shown in Fig. 2 and 3. Low external Ca^{++} reduced the responsiveness of each portion of the vascular bed and shifted the ED_{50} for NA to the right (toward higher concentrations of NA) in each section.

However quantitatively the effects of low Ca^{++} on the NA concentration-effect curves differed considerably in the consecutive segments of the vasculature, which could be directly

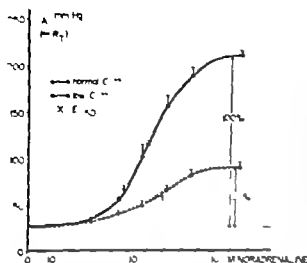


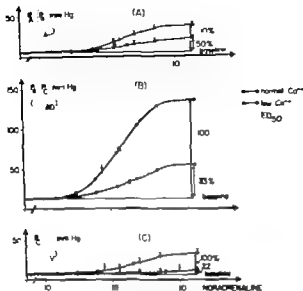
Fig. 3. Concentration-effect curve of adrenaline on arterial inflow pressure in isolated, perfused rat hindquarter normal or low calcium in the perfusate. Vertical dashed line indicates a steady concentration of $7.5 \cdot 10^{-7}$ M the horizontal dashed line demarcated to answer how much the noradrenaline concentration must be increased at low calcium to do the same pressure response. The individual x and vertical bars represent $M \pm SE$ of 41–19 animals.

respectively (cf Follom *et al.* 1974, Elvasen *et al.* 1974). Knowing R_A and R_{AP} the resistance of distal precapillary resistance vessels (R_{AD}) could be deduced as well. The resistance drop along vascular tree is schematically shown in Fig. 1 which also indicates in general principle the region which measurement were made or calculated.

Pressure responses to NA at concentrations in the perfusion fluid from $1.5 \cdot 10^{-7}$ to 10^{-5} M, measured at low (0 mM) and at normal (1.5 mM) Ca^{++} concentration in the perfusate. Concentration-effect curves for NA were then constituted for pressure responses of the total vascular bed and the consecutive segments of the vascular bed, i.e. the "proximal" and "distal" precapillary resistance vessels and the postcapillary resistance vessels. Since flow was kept constant the changes in pressure drop of these consecutive segments reflect directly the respective changes in their resistances (Fig. 2).

Results

The resistance to flow offered by the "proximal" resistance vessels (R_{AP}) makes up about 25% of R_T in the perfused hindquarter vascular bed during maximal vasodilatation; about 20% during maximal constriction, as long as Ca^{++} is normal (Fig. 3 compared Fig. 3 A). To show that this relatively high contribution of R_{AP} to R_T during constriction is not merely due to the circumstances of perfusion, with a diffuse distribution of high concentrations and a consequent artificially intense involvement of proximal "resistance arteries" the changes in $P_A - P_V$ also were followed when the rats were still intact with normal blood perfusion and vasoconstrictor fibre control. During basal *in vivo* conditions $P_A - P_V$ accounted for some 10% of the total systemic pressure drop, implying that "distal" precapillary resistance vessels (R_{AD}) then contributed 70–75% if it is assumed that mean capillary pressure is around 15 mmHg in this situation (cf Elvasen *et al.* 1974) and central venous pressure close to zero. However the contribution to total pressure drop ($P_A - P_V$) of $P_A - P_V$ increased from about 10% to some 45% upon intense vasoconstriction, which was elicited as a reflex response to graded withdrawal of blood. Since flow in the steady state situation is always the same in the consecutive segments of a vascular circuit, it follows from this observation that the "proximal" resistance vessels of the rat are relatively more strongly constricted by the vasoconstrictor fibres than the "distal" or "resistance arteries". Thus the observations during hindquarter perfusion with plasma substitute and noradrenaline



3 Legends as for Fig. 2, except: A) $P - P_A - P$ is pressure measured in a small artery with an external radius complete relaxation (when exposed to locally applied papaverine) around 0.1-0.15 (see text). $P_A - P$ is proportional to R_{AP} ("proximal" precapillary resistance) since flow is kept constant throughout. Its total vertical bars represent $M \pm S.E.$ from 6 animals. B) $P - P$ is proportional to R_{AD} ("distal" capillary resistance). The points are values calculated from the curves shown in Fig. 2, 3A and 3C. $P - P$ is proportional to postcapillary venous resistance. The points represent $M \pm S.E.$ from 8 or 9 animals. As in Fig. 2 the vertical dashed line in A, B and C indicates noradrenaline concentration of $10^{-4} M$. The horizontal dashed line demonstrated by arrows indicates how much the noradrenaline concentration must be increased at low calcium to elicit the same pressure response.

Infusion coincide closely with observations in the intact rat as to the relative magnitude of the resistance increases in the "proximal" resistance vessels. They imply that the normal control of resistance is not only localized to the distal microvessels but involves relatively extensive portions of the precapillary vessels, including their larger proximal ends as well. There is also clear tendency for functional differentiation among the proximal and distal vessels in the rat. The proximal resistance vessels evidently contribute relatively more to the centrally directed vasoconstrictor fibre control than to the locally originated myogenic resistance, which consequently resides mainly in the distal precapillary resistance vessels under basal conditions as was shown, of course, with a considerable spatial overlap. Concerning the situation during *in vitro* perfusion the concentration of Ca^{++} in the venous effluent was from 0.4 to 0.5 mM during perfusion with low Ca^{++} solutions (0.2 mM). The prior responses to increasing concentrations of NA in consecutive segments of the vascular bed, perfused with normal or low Ca^{++} solutions, are shown in Fig. 2 and 3. Low external Ca^{++} reduced the responsiveness of each portion of the vascular bed and shifted the ED₅₀ for NA to the right (toward higher concentrations of NA) in each section.

However quantitatively the effects of low Ca^{++} on the NA concentration-effect curves differed considerably in the consecutive segments of the vasculature, which could be directly

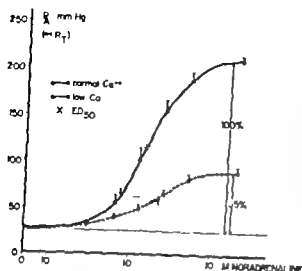


Fig. 2. Concentration-effect curve of adrenaline on arterial inflow pressure P_A in isolated perfused rat hindquarters: normal or low calcium in the perfusate. Vertical dashed line indicates a secondary concentration of $7.5 \cdot 10^{-6}$ M; the lower dashed line demarcated by arrows indicates how much the noradrenaline concentration must be increased at low calcium to elicit same pressure response. The individual bars and vertical bars represent $M \pm S.E.$ 4 to 13 animals.

respectively (cf Folkow *et al* 1974, Eliassen *et al* 1974). Knowing R_A and R_{AP} , the resistance of "distal" precapillary resistance vessels (R_{AD}) could be deduced as well. The resistance drop along the vascular tree is schematically shown in Fig. 1 which also indicates in general principle the segments in which measurements were made or calculated.

Pressure responses to NA, at concentrations in the perfusion fluid from $1.5 \cdot 10^{-6}$ to $2 \cdot 10^{-4}$ M, were measured at low (0.5 mM) and 1 normal (1.5 mM) Ca^{++} concentration in the perfusate. Concentration-effect curves for NA were then constituted for pressure responses of the total vascular bed and for its consecutive segments of the vascular bed, i.e. the "proximal" and "distal" precapillary resistance vessels and the postcapillary resistance vessels. Since flow was kept constant the changes in pressure drop along these consecutive segments reflect directly the respective changes in their resistances to flow.

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	(1) P _A			(2) P - P			(3) P - P			(4) P - P		
	At ED ₅₀	At 2.5 10 ⁻⁴ M		At ED ₅₀	At 2.5 10 ⁻⁴ M		At ED ₅₀	At 2.5 10 ⁻⁴ M		At ED ₅₀	At 2.5 10 ⁻⁴ M	
normal	100	100		100	100		100	100		100	100	
per	34	29		50	48		33	26		19	9	

ner (P) responses to noradrenaline at ED₅₀ and at 2.5 10⁻⁴ M. (Response in normal Ca⁺⁺ = 100.)
 The ED₅₀ responses allow comparisons to be made at the same point on the concentration-effect curve,
 not necessarily at the same concentration of noradrenaline, since low concentrations of calcium
 shift the ED₅₀ toward higher concentrations of noradrenaline. The concentration of 2.5 10⁻⁴ M was
 used since this is on the linear part of the concentration-effect curve. Regardless of which type of
 response is made the largest, proximal resistance vessels (P_A - P_D) retain their responses to noradrenaline
 far in excess of the distal precapillary resistance vessels (P - P_D) or the postcapillary resistance
 vessels (P - P₂).

Discussion

Our results clearly show that lowering Ca⁺⁺ in the perfusate reduces the responses to NA to
 much greater extent in the microvessels of the periphery than in the larger proximal pre-
 capillary vessels and this in spite of the likelihood that the actual concentration of Ca⁺⁺
 in the perfusate gradually becomes higher towards the microvascular level, presumably be-
 cause Ca⁺⁺ is leached out of the tissues during perfusion with low Ca⁺⁺ concentrations. In
 a previous experiment for example, Ca⁺⁺ concentration was in most cases 4-0.5 mM opposed
 to an inflow concentration of 0.2 mM. This increasing Ca⁺⁺ concentration of the perfusate
 after equilibration with the tissues would particularly affect the microvessels placed close
 to the capillary level.

These *in vivo* findings that dependence on external calcium for vascular smooth muscle
 contractility to NA increases as one proceeds from central to peripheral vessels are con-
 sistent with similar suggestions based on comparisons of the canine aorta, carotid and
 mesenteric arteries in an *in vitro* study of the effects of neomycin on Ca⁺⁺ binding and distri-
 bution (Goodman *et al.* 1975). It should further be stressed that in most vascular circuits,
 the smallest precapillary resistance vessels normally display a particularly pronounced myo-
 genic tone, and these spontaneous contractions must reasonably also be highly dependent
 on Ca⁺⁺ influx. In fact, they are presumably even more dependent on extrinsic Ca⁺⁺ than
 when NA induces contractions because NA appears to facilitate the mobilization of Ca⁺⁺.
 It is therefore likely that this hemodynamically most important inherent tone of the smallest
 precapillary resistance vessels is particularly much depressed by reductions in Ca⁺⁺ con-
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It is of primary interest to know which of the several types of larger vessels commonly
 used *in vivo* that constitutes the most appropriate model of microvascular sections respon-
 sible for resistance, capillary flow distribution and capacitance, including dependence
 on external Ca⁺⁺. Two commonly used vessels for *in vitro* studies are rat thoracic aorta and
 portal vein. In such characteristics as inherent activity, propagation of excitation and con-

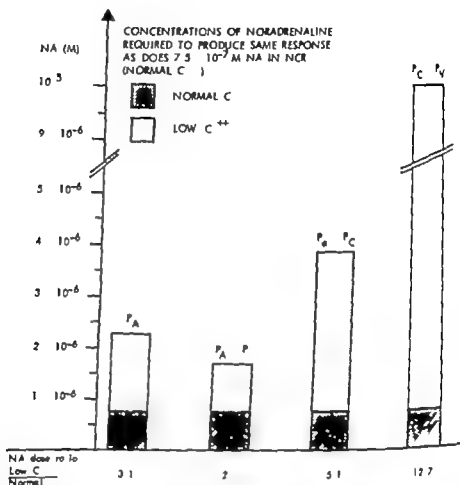


Fig. 4. Comparison of the concentrations of noradrenaline which are equipressor in hindquarters perfused with normal or low calcium solutions. P_A represents the pressor (resistance) changes in the hindquarters vascular circuit as a whole, $P_A - P$ and $P_A - P_O$ those in the proximal and distal precapillary resistance vessels, respectively and $P_O - P_V$ those in the postcapillary resistance vessels.

estimated from the pressure drops since flow was kept constant (Fig. 4 and Table I). Fig. 4 shows the NA concentrations which are equipressor in low and normal Ca^{++} perfusions and the ratios of one to the other. A comparison of these ratios shows that a reduced external calcium concentration leads to a far more extensive depression of the NA responses in the immediately pre- and postcapillary segments ($P - P_O$ and $P_O - P_V$) than in the larger vessels ($P_A - P$). Thus, the NA concentration need only be doubled to produce the same pressor response in the proximal precapillary vessels, whereas it must be increased some 5- to 14-fold to produce equipressor responses in the distal small pre- and postcapillary vessels, respectively.

Table I compares pressor responses to NA with low vs. normal Ca^{++} in the perfused hindquarters at two points in the concentration effect curves: at the ED_{50} and at an arbitrary NA concentration of $2.5 \cdot 10^{-6}$ M. Again, low Ca^{++} is seen to reduce the responses to NA considerably more in the small pre- and postcapillary segments than in the larger upstream vessels (Columns (2) vs. (3) or (4)).

		(1)		(2)		(3)		(4)	
		P		P _A -P		P-P		P _G -P _V	
		At	At	At	At	At	At	At	At
		ED ₅₀	2.5 10 ⁻⁶ M	ED ₅₀	2.5 10 ⁻⁶ M	ED ₅₀	2.5 10 ⁻⁶ M	ED ₅₀	2.5 10 ⁻⁶ M
normal	100	100		100	100	100	100	100	100
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nor (P) responses to noradrenaline at ED₅₀ and at 2.5 10⁻⁶ M. (Response is normal Ca⁺⁺ = 100.) In ED₅₀ responses allow comparisons to be made at the same point on the concentration-effect curve, not necessarily at the same concentration of noradrenaline, since low concentrations of calcium (at ED₅₀) toward higher concentrations of noradrenaline. The concentration of 2.5 10⁻⁶ M was used since this is on the lower part of the concentration-effect curve. Regardless of which type of species, since the larger proximal resistance vessels (P_A-P_G) retain their response to noradrenaline for as low calcium than the distal precapillary resistance vessels (P-P_G) or the postcapillary resistance vessels (P-P).

Discussion

It results clearly show that lowering Ca⁺⁺ in the perfusate reduces the responses to NA to much greater extent in the microvessels of the periphery than in the larger proximal precapillary vessels and this in spite of the likelihood that the actual concentration of Ca⁺⁺ in the perfusate gradually becomes higher towards the microvascular level, presumably because Ca⁺⁺ is leached out of the tissues during perfusion with low Ca⁺⁺ concentrations. In a venous effluent for example, Ca⁺⁺ concentration was in most cases 0.4-0.5 mM opposed to an inflow concentration of 0.2 mM. This increasing Ca⁺⁺ concentration of the perfusate after equilibration with the tissues would particularly affect the microvessels placed close to the capillary level.

These *in vivo* findings that dependence on external calcium for vascular smooth muscle contractility to NA increases as one proceeds from central to peripheral vessels are consistent with similar suggestions based on comparisons of the canine aorta, carotid and mesenteric arteries in an *in vitro* study of the effects of neomycin on Ca⁺⁺ binding and distribution (Goodman *et al.* 1975). It should further be stressed that in most vascular districts, the smallest precapillary resistance vessels normally display a particularly pronounced myogenic tone, and these "spontaneous" contractions must reasonably also be highly dependent on Ca⁺⁺ influx. In fact, they are presumably even more dependent on extrinsic Ca⁺⁺ than when NA induces contractions because NA appears to facilitate the mobilization of Ca⁺⁺. It is therefore likely that this hemodynamically most important inherent tone of the smallest precapillary resistance vessels is particularly much depressed by reductions in Ca⁺⁺ concentration.

It is of primary interest to know which of the several types of larger vessels commonly used *in vitro* that constitutes the most appropriate model of microvascular sections responsible for resistance, capillary flow distribution and capacitance, including dependence on external Ca⁺⁺. Two commonly used vessels for *in vitro* studies are rat thoracic aorta and portal vein. Both characteristics as inherent activity propagation of excitation and con-

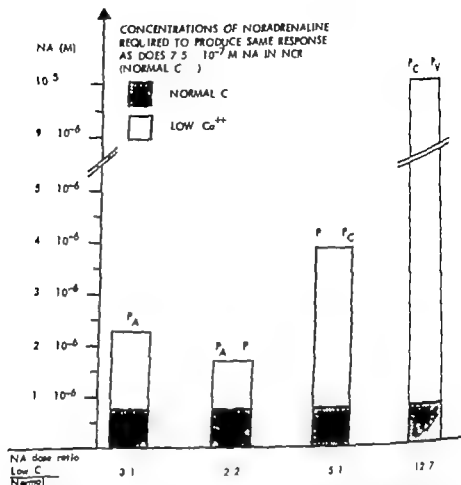


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1

(1) P			(2) P → P			(3) P → P			(4) P ₀ → P _y		
At	At		At	At		At	At		At	At	
ED ₅₀	2.5	10 ⁻⁴ M	ED ₅₀	2.5	10 ⁻⁴ M	ED ₅₀	2.5	10 ⁻⁴ M	ED ₅₀	2.5	10 ⁻⁴ M
mmHg	100	100	100	100		100	100		100	100	
	34	29	50	48		33	26		19	9	

the (P) responses to norepinephrine at ED₅₀ and at 2.5 · 10⁻⁴ M. (Response at normal Ca⁺⁺ = 100.)
 The ED₅₀ responses allow comparisons to be made at the same point on the concentration-effect curve,
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 portal vein. In such characteristics as inherent activity propagation of excitation and con-

striator fibre influence the smooth muscle of the microvessels appears to be more related to that of the portal vein than to that of the aorta (e.g. Ljung 1970, Rhodes and Sutter 1971). Concerning the dependence on external Ca^{++} it has been found (Sutter 1976) that the presence of 0.4 mM external Ca^{++} aortic strips retain 90% and portal veins only 40% of their maximal response to NA in normal Ca^{++} . It seems reasonable to suppose that 0.4 mM Ca^{++} is similar to the concentration of Ca^{++} achieved in our hindquarter fusions since the inflow and outflow concentrations were 0.2 and 0.4–0.5 mM, respectively. The portal vein in its behaviour in low Ca^{++} then is remarkably similar to the distal precapillary resistance vessels (here corresponding to P_{-P_0}) which retain 33% of the maximal responses to NA and also similar to the postcapillary resistance vessels (P_{+P_0}) (Table I column 3). It seems that the mesenteric-portal vein is in several important respects a better analogue of the true resistance vessels than is aortic smooth muscle, as has been suggested previously (Ljung 1970, Rhodes and Sutter 1971) and according to the present findings this is true also with regard to sources of Ca^{++} . Furthermore at any given low Ca^{++} the myogenic contractions of the portal vein are far more depressed than those induced by NA (Sutter 1976). This is in line with the suggestion mentioned above that precapillary myogenic tone, which normally seems to be the dominant element in the maintenance of the systemic resistance to flow is likely to be particularly depressed by reductions of Ca^{++} in the immediate chemical environment.

The studies were supported by grants from the Swedish Medical Research Council (No 14X-80), British Columbia Heart Foundation and the British Medical Research Council (J. V. Jones is an MRC Travelling Research Fellow for 1975–76). — The expert technical assistance of Gunnel Andersson, Ulla Axelsson and Gertrud Karlsson is gratefully acknowledged.

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Time Course and Extent of Carotid Sinus Baroreceptor Threshold Resetting in Rats with Renovascular Hypertension

By

JOHN V. JONES

Received 1 July 1976

Abstract

J. V. Time course and extent of carotid sinus baroreceptor threshold resetting in rats with renovascular hypertension. *Acta physiol. scand.* 1977 99 173-182.

by the extent and time course of arterial baroreceptor threshold resetting so increases in blood pressure in hypertension was induced in young normotensive male Wistar rats by unilateral renal artery clasp. At different intervals after operation the extent of baroreceptor threshold resetting in the animals was examined. Experiments were performed 7, 14 and 25 days after renal artery clamping to baroreceptor resetting to be correlated with the rise and extent of cardiovascular changes in rats with renovascular hypertension of idiopathic duration (Landgren *et al.* 1974). Baroreceptor thresholds in the carotid sinuses were established by progressive clamping of both carotid arteries after partial resection of the sinuses. The results show that after one week of hypertension baroreceptor resetting is just apparent but by two weeks resetting is gross and seems to be largely completed. This parallels the changes in the haemodynamics of renal hypertensive rats and it is concluded that baroreceptor resetting is a secondary phenomenon related to the structural changes induced in the vessels by the elevated pressure.

There is general agreement that the arterial baroreceptors are reset to the higher blood pressure levels in hypertensive animals and man. This was clearly demonstrated in dogs with renal hypertension by McCubbin, Green and Page (1956) and has been abundantly confirmed since then (Aars 1968, Angel-James 1973) in a variety of species including spontaneously hypertensive rats (Nosaka and Wang 1972, Saper and Wang 1976). Various different mechanisms have been postulated to account for baroreceptor resetting and include altered excitability of the receptor area (Aars 1969), degeneration of the receptor endings (Kozdil 1971, Angel-James 1973) or combination of the two. Central mechanisms may of course, be important in the overall picture of resetting but do not explain alterations in impulse traffic in the carotid sinuses and aortic nerves themselves.

Resetting of aortic baroreceptors may occur extremely rapidly in acutely induced hypertension (Krieger 1970). It is also present in carotid sinus baroreceptors a few days after the onset of renal hypertension in dogs (McCubbin 1958). However the exact time course of this

Present address: University Department of Medical Cardiology Royal Infirmary Glasgow Scotland.

striator fibre influence the smooth muscle of the microvessels appears to be more related to that of the portal vein than to that of the aorta (e.g. Ljung 1970, Rhodes and Sutter 1971). Concerning the dependence on external Ca^{++} it has been found (Sutter 1976) that in the presence of 0.4 mM external Ca^{++} aortic strips retain 90% and portal veins only 40% of their maximal response to NA in normal Ca^{++} . It seems reasonable to assume that 0.4 mM Ca^{++} is similar to the concentration of Ca^{++} achieved in our hindquarter fusions since the inflow and outflow concentrations were 0.2 and 0.4–0.5 mM, respectively. The portal vein in its behaviour in low Ca^{++} then is remarkably similar to the precapillary resistance vessels (here corresponding to $P_{\text{a}} - P_{\text{v}}$) which retain 33% of maximal responses to NA and also similar to the postcapillary resistance vessels (Table I column 3). It seems that the mesenteric-portal vein is in several important respects a better analogue of the true resistance vessels than is aortic smooth muscle, as has been suggested previously (Ljung 1970, Rhodes and Sutter 1971), and according to the present findings this is true also with regard to sources of Ca^{++} . Furthermore, at any given low Ca^{++} the myogenic contractions of the portal vein are far more depressed than those induced by NA (Sutter 1976). This is in line with the suggestion mentioned above that precapillary myogenic tone, which normally seems to be the dominant element in the maintenance of the systemic resistance to flow, is likely to be particularly depressed by reductions of Ca^{++} in the immediate chemical environment.

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Abstract

J V Time course and extent of carotid sinus baroreceptor threshold resetting in rats with renovascular hypertension. Acta physiol. scand. 1977 99 173-182.

The extent and time course of arterial baroreceptor threshold resetting to increases in blood pressure in hypertension was studied in young normotensive male Wistar rats by unilateral renal artery ligation. At different intervals after operation the extent of baroreceptor threshold resetting in the same animals was estimated. Experiments were performed 7, 14 and 21 days after renal artery clipping to determine whether baroreceptor resetting to be correlated with the rate and extent of cardiovascular changes in rats with renovascular hypertension of identical duration (Lundgren *et al.* 1974). Baroreceptor threshold resetting was established by progressive clamping of both carotid arteries after partial resection of the sinuses. The results show that after one week of hypertension baroreceptor resetting had appeared but by two weeks resetting is gross and seems to be largely completed. This parallels gross changes in the haemodynamics of renal hypertensive rats and it is concluded that baroreceptor resetting is a secondary phenomenon related to the structural changes induced in the vessels by the elevated pressure.

It is general agreement that the arterial baroreceptors are reset to the higher blood pressures in hypertensive animals and man. This was clearly demonstrated in dogs with renal hypertension by McCubbin, Green and Page (1956) and has been abundantly confirmed since (Aars 1968, Angell-James 1973) in a variety of species including spontaneously hypertensive rats (Nomura and Wang 1972, Sapiro and Wang 1976). Various different mechanisms have been postulated to account for baroreceptor resetting and include altered distensibility of the receptor area (Aars 1969), degeneration of the receptor endings (Kendall and Angell-James 1973) or a combination of the two. Central mechanisms may of course, be important in the overall picture of resetting but do not explain alterations in impulse frequency in the carotid sinus and aortic nerves themselves.

Resetting of aortic baroreceptors may occur extremely rapidly in acutely induced hypertension (Krieger 1970). It is also present in carotid sinus baroreceptors a few days after the onset of renal hypertension in dogs (McCubbin 1958). However the exact time course of this

¹ Present address: University Department of Medical Cardiology Royal Infirmary Glasgow Scotland.

resetting in relation to the development of chronic hypertension is unknown. It is clear to what extent resetting is secondary to structural vascular changes consequent to the hypertension.

In this study the time course and extent of carotid sinus baroreceptor threshold has been studied in rats with experimental renovascular hypertension. In such rats the time course and extent of blood pressure rise and the secondary cardiovascular structural changes following renal artery clipping are known (Lundgren *et al.* 1974). It should therefore be possible to correlate in such animals the extent of structural vascular adaptation with the extent of baroreceptor resetting.

Methods

In order to produce renovascular hypertension in 6-7 week old male Wistar rats, a silver clip (#2, 1 mm internal diameter) was placed on the left renal artery under brief ether anaesthesia. The right kidney was left intact. In this way about 50 per cent of the operated animals developed hypertension of sufficient degree as to be included in this study. Animals were not considered to be hypertensive unless their mean blood pressure in excess of 145 mmHg.

On the day of the baroreceptor threshold study the degree of hypertension was determined by intraarterial blood pressure measurement in the caudal artery. The tail artery was cannulated under ether anaesthesia after which the arterial blood pressure and heart rate were measured when the animal was fully awake. The heart rate and blood pressure in a control group of animals were determined in the identical way. The caudal artery was also used for measurement of the systemic arterial pressure in the experiment. Pressures were recorded by means of Statham pressure transducers and all data were recorded on a Grass model 7 polygraph.

A control group of animals was studied as were animals with renovascular hypertension of 25 days duration. 6 animals were included in each group.

After determination of awake heart rate and blood pressure the animals were then anaesthetized with ether. The right femoral vein was cannulated and chloralose (60 mg/kg) administered intravenously whereupon the ether was discontinued. The animal was placed on its back and all four limbs held down. A midline incision was made from mandible to sternum. The skin and subcutaneous tissues were retracted and a tracheal cannula was inserted. The trachea and oesophagus were dissected free from the tracheal cannula and retracted. This allowed easier access to the carotid bifurcation on both sides depending on the direction of retraction of the trachea and oesophagus. Both vagus nerves were dissected free were both hypoglossal nerves and hyoid bones. This latter maneuver provided greater access to the external carotid arteries which were then both cannulated and connected to pressure transducers. All branches of the external carotid arteries were ligated but great care was taken to avoid damage to the carotid bifurcation region and themselves. The internal carotid and occipital arteries were left intact and undisturbed. The common carotid arteries were exposed on both sides and freed from the carotid sinus regions as possible. The vagi and cervical sympathetic trunks were left intact. Clamps were placed round the common carotid arteries on both sides. By tightening the clamps the distal pressure in the clamps could be reduced and the new pressure levels recorded in the carotid bifurcation. The extent to which the carotid sinus pressure could be lowered depended upon the degree of retrograde filling of the sinus. In the event that no animal was retrograde filling so great as to allow the determination of a threshold for carotid sinus baroreceptor activation, i.e. the pressure in the sinus could always be lowered sufficiently despite the internal carotid artery being left intact.

The animals were paralysed with gallamine (10 mg/kg i.v.) and put on intermittent positive pressure ventilation by means of a Starling pump. The animal was ventilated with gas containing 10% CO₂ in O₂. Anaesthesia was maintained where necessary with intravenous chloralose and additional doses (10 mg/kg i.v.) was given every 20 min. The animals were allowed to stabilize for 30 min after completion of the surgery before measurement of the baroreceptor threshold was made.

Carotid sinus pressure was reduced in steps of approximately 20 mmHg by adjustment of the artery clamps. As clamping of one carotid artery commonly caused rise of blood pressure and to measure the opposite sinus pressure some manipulation of the clamps was necessary before

Mean values \pm S.E. for renal hypertensive rats (RHR) 7, 14 and 25 days after renal artery constriction and a group of normotensive controls (NCR) concerning body weight, awake arterial blood pressure, left ventricular weight and percentage left ventricular weight/body weight

	Arterial blood pressure, mmHg	Body weight, g	Left ventricular weight, g	Percentage left ventricular weight/body weight
	94.0 \pm 3.9	235 \pm 14.6	0.90 \pm 0.033	0.215 \pm 0.008
days	157.3 \pm 1.9	214 \pm 8.0	0.55 \pm 0.033	0.254 \pm 0.011
4 days	163.6 \pm 3.5	230 \pm 7.3	0.64 \pm 0.021	0.282 \pm 0.007
5 days	166.0 \pm 4.0	271 \pm 8.6	0.74 \pm 0.026	0.287 \pm 0.003

stable mean pressure could be obtained. In addition the rise in blood pressure consequent upon the arterial pressure reduction often caused a secondary rise in aortic pressure with some oscillation of blood pressure as a result. This was particularly noticeable in the first step where blood pressure rose most to its greatest. The aortic pressure and blood pressure were allowed to stabilize at a new level for as long as it took to obtain reasonably settled values. This was usually at least one minute and often longer. Aortic pressures at each step were adjusted to be as near each other as possible although this proved difficult to have the pressures exactly the same. Pressure differences between the two aortas were therefore accepted provided they were not greater than 10 mmHg. The step-wise reduction in pressure was repeated several times in each animal to allow confirmation of the threshold pressure in each.

At the end of each experiment the hearts of all the animals were excised and the left ventricles dissected and weighed separately. The mean wet ventricular weight for each group was then calculated as was the average left ventricular weight/body weight.

Results

Arterial blood pressures measured in the tail artery during awake conditions are presented in Table I. Within one week of the operation, renal hypertensive rats show a significant increase in blood pressure when compared to control animals. Thereafter the blood pressure increases only slightly further. When comparing left ventricular weights as a percentage of body weight they are significantly increased after one week of hypertension with a further increase after 14 days by which time they seem to have reached a stable value. Thus these results parallel those of Lundgren *et al.* (1974).

The response of the arterial blood pressure to changing the mean carotid sinus pressure is shown for all 4 rats in Fig. 1-4. When carotid sinus pressure is lowered arterial blood pressure falls. Subsequently as the sinus pressure is reduced to threshold values and lower there are no further increases in blood pressure. However because of the wide individual variation in blood and sinus pressures, for statistical evaluation of the results, the blood pressure attained in each animal at each step was expressed as a percentage of the maximum blood pressure attained in each animal, i.e. as a percentage of the blood pressure at the threshold value for baroreceptor activation. Table II shows the results obtained in this way at each group using 2 separate pressure response curves in each animal. Mean carotid sinus pressure has been grouped in 10 mmHg blood pressure intervals and the percentage blood pressures compared, using the *t*-test, with the blood pressure group in the lowest sinus pressure range of each group. In this way the threshold pressure for carotid sinus baroreceptor activation in the normotensive group of rats was found to be in the range 70-84 mmHg. In

resetting in relation to the development of chronic hypertension is unknown. It is not clear to what extent resetting is secondary to structural vascular changes consequent to the hypertension.

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	Arterial blood pressure, mmHg	Body weight, g	Left ventricular weight, g	Percentage left ventricular weight/body weight
	99.0 ± 3.9	235 ± 14.6	0.50 ± 0.033	0.213 ± 0.008
7 days	157.3 ± 1.9	214 ± 8.0	0.53 ± 0.033	0.258 ± 0.011
14 days	165.6 ± 3.3	230 ± 7.3	0.64 ± 0.021	0.282 ± 0.007
21 days	164.0 ± 6.0	271 ± 8.6	0.74 ± 0.026	0.287 ± 0.003

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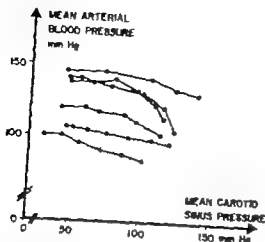


Fig. 1

Fig. 1 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in normotensive control rats (NCR).

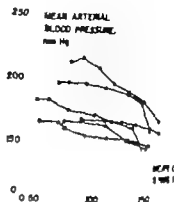


Fig. 2

Fig. 2 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 7 days after renal artery constriction.

rats with renovascular hypertension of one weeks duration the threshold was in the sinus pressure range 85-99 mmHg. There was thus a slight resetting of the baro threshold in these rats when compared to normotensive control animals. However, a group of rats in whom the renal artery had been clipped 14 days previously the threshold for carotid sinus baroreceptor activation was much higher being in the range 145-159 mmHg. A similar threshold pressure range (145-159 mmHg) was found in rats with renovascular hypertension of 25 days duration. Thus baroreceptor resetting seems to be largely complete by 2 weeks, a time course that parallels the structural vascular and cardiac changes in the same species following production of renovascular hypertension. Although the threshold

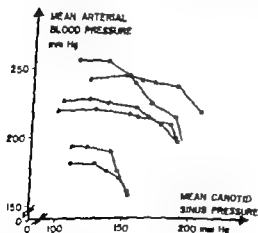


Fig. 3

Fig. 3 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 14 days after renal artery constriction.

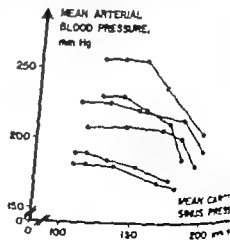


Fig. 4

Fig. 4 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 25 days after renal artery constriction.

Mean values \pm S.E. for renal hypertensive rats (RHR) 7, 14 and 25 days after renal artery constriction and group of normotensive controls (NCR) concerning percentage maximum blood pressure attained in each animal. C.S.P. = carotid artery pressure.

mmHg	NCR	Percentage maximum blood pressure		
		RHR 7 days	RHR 14 days	RHR 25 days
	99.8 \pm 0.1	—	—	—
	98.7 \pm 0.4	99.9 \pm 0.1	—	—
	96.6 \pm 0.9	99.2 \pm 0.3	—	—
	p 0.001	ns		
	93.1 \pm 1.0	97.4 \pm 0.9	—	—
	p 0.001	p 0.001		
	90.2 \pm 1.2	95.2 \pm 1.2	99.0 \pm 0.3	99.7 \pm 0.3
	p 0.001	p 0.001		
	88.8 \pm 2.1	93.3 \pm 1.3	99.4 \pm 0.3	100 \pm 0.0
	p 0.001	p 0.001	ns	ns
	—	89.6 \pm 1.3	98.6 \pm 0.4	99.0 \pm 0.6
		p < 0.001	ns	ns
	—	83.0 \pm 2.3	94.3 \pm 1.2	96.7 \pm 0.8
		p < 0.001	p < 0.01	p < 0.001
	—	—	92.5 \pm 2.1	93.8 \pm 1.4
			p 0.001	p < 0.001
	—	—	91.7 \pm 1.7	90.0 \pm 2.0
			p 0.001	p 0.001
	—	—	84.9 \pm 2.1	83.8 \pm 2.6
			p 0.001	p 0.001

d to be reset to higher levels when the blood pressure elevation was greater this is a reason for the threshold differences observed between the 7-day hypertensive group and 14 and 25-day hypertensive groups. Fig. 5 shows pressure responses from animals under resting awake blood pressures but with hypertension of different duration. In animal with hypertension of 7 days duration the threshold lies between 93 mmHg and 100 mmHg while the threshold in the animal with hypertension of 14 days duration lies between 137 mmHg and 125 mmHg.

Discussion

Baroreceptors of both the carotid sinus and the aortic arch have been extensively investigated in normotensive animals (Kirchheim 1976). To a lesser extent they have been studied in hypertensive animals including renal hypertensive dogs (McCubbin *et al.* 1956), rabbits (1968, Angell-James 1973) and spontaneously hypertensive rats (Nosaka and Wang 1976, Sapru and Wang 1976) and also in man (Brinow *et al.* 1969, Korner *et al.* 1974). There is almost unanimous agreement that the entire pressure response curves of baroreceptor function in hypertensive animals are reset to respond in the higher levels of mean arterial blood pressure although this may not be true for the spontaneously hypertensive rats (Nosaka and Okamoto 1970).

There is little agreement about the mechanism of resetting of the baroreceptors. Some

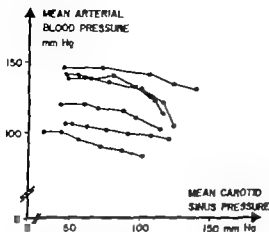


Fig. 1

Fig. 1 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in normotensive control rats (NCR)

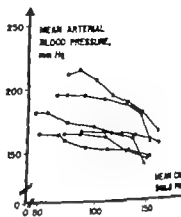


Fig. 2

Fig. 2 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 7 days after renal artery constriction.

rats with renovascular hypertension of one weeks duration the threshold was in the sinus pressure range 85-99 mmHg. There was thus a slight resetting of the baroreceptor threshold in these rats when compared to normotensive control animals. However, a group of rats in whom the renal artery had been clipped 14 days previously the threshold for carotid sinus baroreceptor activation was much higher being in the range 145-159 mmHg. A similar threshold pressure range (145-159 mmHg) was found in rats with renal hypertension of 25 days duration. Thus baroreceptor resetting seems to be largely complete by 2 weeks, a time course that parallels the structural vascular and cardiac changes in the same species following production of renovascular hypertension. Although the

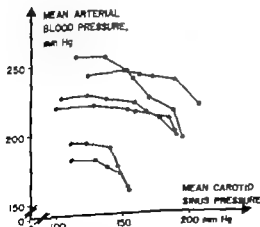


Fig. 3

Fig. 3 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 14 days after renal artery constriction.

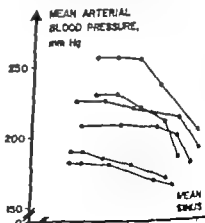


Fig. 4

Fig. 4 The effect on the mean arterial blood pressure of lowering the mean carotid sinus pressure in rats (RHR) 25 days after renal artery constriction

□ Mean values \pm S.E. for renal hypertensive rats (RHR) 7, 14 and 25 days after renal artery constriction and group of normotensive controls (NCR) concerning percentage maximum blood pressure attained in each animal. CSP = carotid sinus pressure.

mmHg	NCR	Percentage maximum blood pressure		
		RHR 7 days	RHR 14 days	RHR 25 days
	99.8 \pm 0.1	—	—	—
	99.7 \pm 0.4	99.9 \pm 0.1	—	—
	ns			
	96.6 \pm 0.9	99.2 \pm 0.5	—	—
	p 0.001	ns		
	93.1 \pm 1.0	97.4 \pm 0.9	—	—
	p 0.001	p 0.001		
4	90.2 \pm 1.2	93.2 \pm 1.2	99.0 \pm 0.3	99.7 \pm 0.3
	p 0.001	p 0.001		
9	86.8 \pm 2.1	93.3 \pm 1.5	99.4 \pm 0.3	100 \pm 0.0
	p 0.001	p 0.001	ns	ns
14	—	89.4 \pm 1.1	98.6 \pm 0.4	99.0 \pm 0.6
		p 0.001	ns	ns
19	—	83.0 \pm 2.3	94.9 \pm 1.2	96.7 \pm 0.8
		p 0.001	p < 0.01	p < 0.001
24	—	—	92.5 \pm 2.1	93.8 \pm 1.4
			p < 0.001	p < 0.001
29	—	—	91.7 \pm 1.7	90.0 \pm 2.0
			p 0.001	p 0.001
34	—	—	84.9 \pm 2.1	83.8 \pm 2.6
			p 0.001	p 0.001

ed to be reset to higher levels when the blood pressure elevation was greater: this is the reason for the threshold differences observed between the 7-day hypertensive group, the 14 and 25-day hypertensive groups. Fig. 5 shows pressure responses from animals similar resting awake blood pressures but with hypertension of different duration. In normal with hypertension of 7 days duration the threshold lies between 110 mmHg and 115 mmHg while the threshold in the animal with hypertension of 14 days duration lies between 137 mmHg and 125 mmHg.

Discussion

Baroreceptors of both the carotid sinus and the aortic arch have been extensively studied in normotensive animals (Kirchheim 1976). To a lesser extent they have been studied in hypertensive animals including renal hypertensive dogs (McCubbin *et al.* 1956), rabbits (1968, Angel-James 1973) and spontaneously hypertensive rats (Nomaka and Wang 1976, Saxena and Wang 1976) and also in man (Bristow *et al.* 1969, Komer *et al.* 1974). There is almost unanimous agreement that the entire pressure response curves of baroreceptor function in hypertensive animals are reset to respond to the higher levels of mean arterial blood pressure although this may not be true for the spontaneously hypertensive (Nomaka and Okamoto 1970).

There is little agreement about the mechanism of resetting of the baroreceptors. Some

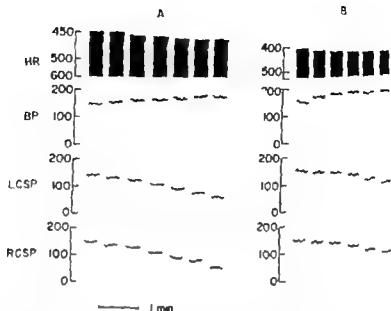


Fig. 5 The effect of lowering mean pressure in both carotid sinuses on the blood pressure (BP) rate (HR) in A—a rat 7 days after renal artery clipping and B—a rat 14 days after renal artery clipping. LCSP—left carotid sinus pressure, RCSP—right carotid sinus pressure.

form of adaptation of the receptor has been postulated. Indeed there is good evidence that the baroreceptor reflex may be centrally altered during sleep (Smyth, Sleight and Hilton 1969), exercise (Sleight *et al.* 1971) and during alertness when defence reactions are aroused (Hilton 1963, Kylstra and Lisander 1970). The majority view of resetting in hypertension, however, favours some form of structural change in either the nerve endings or the regions or both. Aars (1969) has shown less distensibility of the aortic arch in hypertensive rabbits. This altered distensibility results, it is postulated, in splinting of the baroreceptor endings and could account by itself for the resetting in hypertension. However, others have described the presence of degenerating nerve endings in both man (Abrahamson and Berg 1967) and in experimental animals (Angell James 1973). Rees (1968) who has examined the structure of the carotid sinus in some detail with the electron microscope was unable to find any evidence of degenerating nerve endings in the carotid sinuses of dogs with renal hypertension (Sleight *et al.* 1975).

The time course of resetting of the baroreceptors is largely unknown. Krieger (1968) has shown that in rats where the aorta was clamped resetting occurs early in this acute hypertension and the majority of aortic arch baroreceptors have reset within 48 h. He has also shown that reversal of the baroreceptor resetting in rats with renovascular hypertension, where the hypertension is abolished by removal of the renal artery clip, is even more rapid. In this latter study the aortic arch baroreceptors had shown reversal of resetting in a matter of hours. This is, of course, at variance with the majority view where resetting has been attributed to physical changes in either the vessel wall or the baroreceptor themselves. Resetting does appear to occur quite rapidly, however, as it can also be detected within a few days of the onset of renal hypertension in dogs (McCubbin and Page 1968) and rabbits (Aars 1968). The results of the present study indicate that the resetting

Renal baroreceptors has the same time course as the development of structural changes which are themselves secondary to the rise in blood pressure consequent upon clipping one renal artery. It implies that the baroreceptor resetting may be largely secondary to changes although the contribution of central mechanisms cannot be excluded. Lundgren *et al.* (1974) investigated the exact time course and extent of cardiovascular adaptation following the rise in mean arterial blood pressure after induction of renovascular hypertension in normotensive rats. At different time intervals after induction of renovascular hypertension the haemodynamic characteristics of the renal hypertensive and of normotensive control rats were explored using a paired hindquarter perfusion technique. The extent of left ventricular hypertrophy was also examined. It was shown that ventricular hypertrophy was present after one week while adaptive structural changes in resistance vessels were only just appearing. The cardiovascular structural adaptation seemed to be largely completed 2-3 weeks after the operation since no further significant rise in blood pressure, left ventricular hypertrophy or medial hypertrophy occurred at this time. It should be emphasized that under these experimental conditions the blood pressure elevation was comparatively gradual taking 7-14 days to reach a stable maximum.

The time course of the changes in the hindquarter vascular bed appear to parallel the resetting of the baroreceptors in rats with renal hypertension. Resetting was slight, but present, one week when the blood pressure is already grossly elevated and approaching a stable level. At this time structural changes are only just beginning to appear in the resistance vessels although left ventricular hypertrophy is more clearly apparent. The baroreceptors seem to be completely reset after two weeks as there is no difference in threshold values at and 25 days. This also parallels Lundgren *et al.* (1974) findings of the time course of cardiovascular adaptation following renal artery clipping.

It has not been known previously whether the baroreceptors reset passively with the rise in blood pressure in chronic hypertension or whether they precede or follow the rise in blood pressure. Krieger (1970) has shown that following aortic clamping in the rat the aortic baroreceptors first show signs of resetting at about 12 h and most of the fibres examined had reset by 48 h. This does not, of course, exclude a structural basis for the resetting as such gross changes in load might well induce changes in aortic structure within this time or in aortic wall water content with resultant altered distensibility. It is known that skeletal muscle will undergo hypertrophy within this time course in the face of extreme increases in work load (Goldberg 1967). What is more difficult to explain is the observation, again by Krieger (Salgado and Krieger 1973), that the reversal of renal hypertension causes an almost immediate, albeit incomplete, reversal of baroreceptor resetting. This observation would suggest that structural changes may not be so significant at least for the reversal of baroreceptor resetting although if the nerve endings have degenerated it is doubtful whether they could regenerate so rapidly.

McCubbin (1958) has indicated that in chronic hypertension of considerable duration the baroreceptors are tending still to oppose the hypertension. However within the limits of this study the results have shown the baroreceptor threshold to be completely reset after 2 weeks of renovascular hypertension. At this stage the threshold appears to be nearer the awake mean blood pressure of the animals than was the case in the control animals or in those with

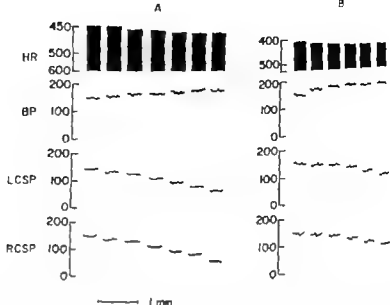


Fig. 5 The effect of lowering mean pressure in both carotid sinuses on the blood pressure (BP) and rate (HR) in A—a rat 7 days after renal artery clipping and B—a rat 14 days after renal artery clipping. LCSP=left carotid sinus pressure RCSP=right carotid sinus pressure.

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ere the superior laryngeal and cervical sympathetic nerves. The contribution of the superior laryngeal nerve was important but variable. In some animals it seemed the major route for baroreceptor afferents while in others section of this nerve had little effect. In the present study the superior laryngeal nerves were sectioned but the cervical sympathetic trunks and vagi were intact. This was the only attempt to denervate the aortic baroreceptors and probably partly explains the variable response to carotid sinus pressure reduction from animal to animal. In consequence it is not possible to describe the sensitivity of the aortic baroreceptors to a given drop in pressure using this technique. Furthermore the clamp used to lower the sinus pressure tended first to diminish the pulse pressure. Thus the initial blood pressure response to carotid artery clamping tended to be the same as not only was the mean carotid sinus pressure reduced but the pulse pressure was also reduced at this step. However none of these factors should affect the result to carotid sinus baroreceptor activation as a measure of resetting of the receptors. In addition it has been shown that resetting of the threshold responses to carotid sinus baroreceptor activation lags behind the development of an elevated blood pressure. Receptors have a similar time course to the development of vascular changes in the perfused hind-limb preparation from rats with renovascular hypertension. The resetting seems to be complete after two weeks and does not, at this stage, appear to oppose the elevated blood pressure. In the initial stages the information from the baroreceptors seems to be overridden by other mechanisms although changes in carotid sinus perfusion pressure are able to impose further changes in blood pressure. Resetting seems to be a secondary phenomenon the time course of which is related to the structural vascular adaptation, itself a response to the raised blood pressure. Thus the results are in agreement with a recent study on spontaneously hypertensive rats (Sapru and Wang 1976) where it was concluded that baroreceptor resetting was secondary to the hypertension and where histological studies showed a close correlation between aortic hypertrophy and baroreceptor resetting.

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hypertension of 1 week's duration. This would tend to suggest that the baroreceptors and carotid sinus are not opposing the hypertension in its chronic phase and may even be facilitating it. Folkow *et al.* (1973) have shown that the same concentration of noradrenaline added to the perfusate in the paired hindquarter perfusions of spontaneously hypertensive rats and normotensive control rats causes greater pressor responses in the former. This "hyperreactivity" is attributed to an altered wall to lumen ratio in the hypertensive animals. Similarly it could be assumed that identical activity of the sympathetic nervous system to the vessels in hypertensive and normotensive animals would lead to greater responses in the former. Therefore an abolition of baroreceptor activity would lead to a greater rebound increase in blood pressure in hypertensive animals compared to normotensive ones. This might explain the apparent difference between the slopes of the response curves in Fig. 1 and 2 and Fig. 3 and 4. A greater increase in blood pressure in response to lowering the sinus pressure could be expected in animals with structural vascular alteration consequent upon their hypertension.

Gribbin *et al.* (1971) have shown that baroreceptor sensitivity declines with increasing age. However it is unlikely that there would be any significant difference in baroreceptor sensitivity between the rats in this study as they were all young animals. Furthermore the control rats were of ages spanning the age and weight ranges of the hypertensive rats and no difference in threshold values was noted between the older normotensive controls and the younger control rats.

Occlusion of both common carotid arteries in the rat is often fatal and is associated with cerebral ischaemia in this species (Thant *et al.* 1969; Fujishima *et al.* 1975). However the technique employed in this study with gradual reduction in carotid sinus pressure and occlusion of the common carotid artery did not seem to produce untoward effects. Furthermore a plateau in the blood pressure response was obtained before total occlusion and when the clamps were closed completely no further effects were noted. This would seem to exclude any chemoreceptor influence on the response as this would also have been progressive with reduction in the sinus pressure. Furthermore the rats were ventilated with 100% pure oxygen, thus minimising chemoreceptor influences while Biscoe, Bradley and Partridge (1970) have shown that chemoreceptor afferent traffic is independent of sinus pressure in the range 60–160 mmHg in cats. In only the normotensive and 7-day hypertensive rats was it necessary to go below this pressure level to obtain a definite threshold for baroreceptor activity. There have, however, been no studies on the relationship between carotid sinus pressure and chemoreceptor discharge in hypertensive animals. Progressive clamping of the carotid arteries could, in theory, have resulted in increased peripheral resistance which might be different in hypertension when compared to normotension. This should, of course, have been progressive until complete occlusion of both common carotid arteries was attained, which was not the response obtained.

Krieger (1964) has suggested that the aortic arch baroreceptors may be dominant over the carotid sinus baroreceptors in circulatory control in the rat. Certainly in attempting to produce neurogenic hypertension in rats both Krieger (1964) and Thant, Yamori and Okamoto (1969), found that aortic baroreceptor denervation was very important in producing a rise in blood pressure. The major routes for afferent traffic from the aortic arch baroreceptors

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Rate-dependent Myogenic Response of Vascular Smooth Muscle during Imposed Changes in Length and Force

By

STEFAN B. SÄGULIDSSON¹, BÖRJE JOHANSSON² and STEFAN MELLANDER¹

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Abstract

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A study of electrical and mechanical responses to stretch in isolated vascular smooth muscle revealed that the rate and graded influence of the rate of change in length, dL/dt (Johansson and Mellander, 1974) regulate dynamic responses at high rates of stretch was much more pronounced than the "static" response to constant, increased ($\sim 40\%$) length. In this previous study the mechanical responses were measured as active force. In view of the fact that myogenic reactions *in vivo* must be associated with changes in caliber, it was considered of interest to investigate the responses to stretch also in smooth muscle during active shortening. In the present study, as in the one referred to above, electrical and mechanical activity in the isolated rat portal vein was studied by the sucrose gap method. However, in the present series the mechanical responses were recorded not only as active force but also as active shortening. A possible comparison of the myogenic responses under these two types of smooth muscle contraction, dynamic passive stretch was found to be associated with marked increases in spike discharge and local activity under both these experimental conditions and the quantitative relation between spike rate and rate of passive stretch (or shortening) is similar. Thus, active shortening of the smooth muscle did not interfere with the ability of the vessel to respond myogenically to passive stretch. A further analysis of the results suggested that, for the preparations as a whole, the dynamic oscillatory response was closely related to the rate of change of passive force, dP/dt , than to dL/dt .

Vascular smooth muscle in resistance and precapillary sphincter vessels is sensitive to stretch and responds to an increase in transmural pressure with enhancement of contractile activity. This kind of response is the background of myogenic reactions in the peripheral circulation, mediated by myogenic autoregulation of blood flow and capillary hydrostatic pressure (see e.g. Folkow 1964; Mellander and Johansson 1968; Johnson 1974). Recent work on the vascular bed of skeletal muscle has indicated that the local vascular responses to rise in transmural pressure are much more vigorous during the dynamic phase of change in pressure than during sustained static pressure elevation (Mellander, Lundvall and Grände 1976; Grände, Lundvall and Mellander 1976), suggesting that the myogenic reaction involves a rate-sensitive element.

The cellular mechanisms behind such dynamic myogenic reactions were elucidated in a series of *in vitro* studies of electrical and mechanical activity in isolated vascular smooth muscle

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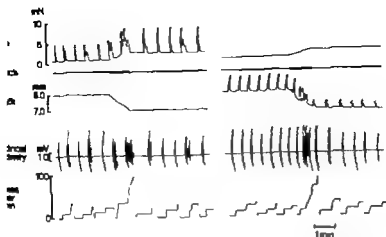


FIG. 1. Effect of dynamic and static passive stretch on mechanically and extracellularly recorded electrical activity of the isolated rat portal vein. In the left panel the muscle was stretched by increasing the length by constant rate. In the right panel the muscle was stretched to the same final length and passive force, by increasing the load at constant rate.

reducer the muscle length (recorded by attaching the isotonic lever to the force transducer), the A.C. recording of the signals from the sucrose-gap electrodes, and the spike counter output. In the control situation with a muscle length of 5.0 mm and preload of 0.7 mN the preparation showed the usual pattern of phasic portal vein contractions associated with bursts of action potentials corresponding to an average of 28 spikes/min. Stretching the preparation by 40% at a rate of 2 mm/min (or about 0.7 per cent total length/s) led to a clear-cut increase in electrical and mechanical activity (spikes/min) increasing to 95 spikes/min during the dynamic stretch. Electrical activity in the 28 period at constant, increased length was only slightly enhanced (to 30 spikes/min) as compared to control activity before stretch. The preparation was then returned to the original length and preload (not shown in the figure).

The right panel of Fig. 1 shows the results obtained in the same muscle under the second experimental condition where the muscle shortened actively during the phasic contractions as shown in the length recording. Mean spike activity in the control period was 27 spikes/min. The muscle was stretched to the same final length and passive force as the left panel but in this case the stretch was applied by increasing the load on the lever at constant dP/dt by means of the spiral spring. The continuous change in force could not be directly recorded under these conditions but was deduced from the calibration of the force transducer and is indicated at the top of the panel. It is evident that this type of passive stretch of a portal vein also led to a significant dynamic excitatory response, spike discharge increasing to 90 spikes/min despite the fact that the muscle could now counteract the passive tension by active shortening. At the later static stage of increased length and passive tension, spike discharge was again only little changed compared to control activity.

(Johansson and Mellander 1975). These authors found pronounced and graded responses to different rates of length changes in preparations of rat portal vein, active effects being observed in response to positive, and inhibitory effects to negative, dL/dt . For instance spike frequency increased to about 300 per cent of control value rates at or above 1.5 per cent of the muscle length per sec. A static increase in length per cent led to only some 15 per cent increase in spike activity. The electrical response associated with enhanced mechanical activity measured as contractile force. In view of the fact that myogenic reactions *in vivo* must lead to changes in vessel caliber in order to adjust hemodynamic parameters, such as resistance to flow. It was considered of importance to investigate the electrical and mechanical responses of the smooth muscle also under conditions which would permit active shortening. The present study is an extension of the earlier work on the isolated portal vein to encompass such mechanical conditions.

A preliminary report on the present study has been published (Sigurdsson, Johansson and Mellander 1976).

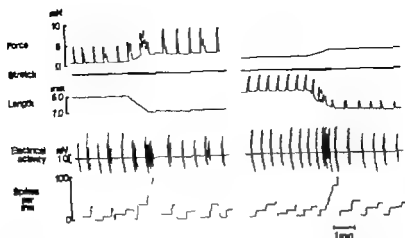
Methods

The portal vein of Sprague-Dawley rats (250–300 g) was dissected and mounted in the sucrose-gap tissue bath as described earlier (Johansson and Mellander 1975). The active part of the preparation, 5–7 mm length, was continuously superfused with standard Krebs solution of the following composition: NaCl 122, KCl 4.73, NaHCO_3 15.5, KH_2PO_4 1.19, MgCl_2 1.19, CaCl_2 2.49, glucose 11.5 and ascorbate 0.026. The solution was bubbled with 4% CO_2 in O_2 giving a pH of 7.3–7.4 and the temperature was kept at 37°C. Electrical activity was studied with the sucrose gap technique (see e.g. Barman and Straub 1958). Electronic counting of the number of spikes was performed as in the earlier study (Johansson and Mellander 1975).

After the muscle had accommodated for at least 1 h at a low preload of about 0.7 mN it was subjected to passive stretch and shortening at graded rates. These changes in length were produced in two ways and two different techniques were used for recording the active mechanical responses. The experimental procedures of the first type were as in the previous study (Johansson and Mellander 1975). The portal vein was connected to a force transducer (Grass FTO3) which could be raised and lowered at grade by means of a micrometer screw. The muscle was thereby exposed to changes in length at selected rates of dL/dt and the contractile responses were recorded as development of active force. In the second type of experimental procedure the muscle was connected to an isotonic lever supplied with a photo device which permitted recording of passive and active changes of muscle length. The load on the lever could be varied in a continuous and controlled manner by a parallel spring connected to the lever. With this arrangement the passive force applied to the muscle could be altered at selected constant rates of dP/dt while the contractile responses were recorded as active shortening. It was possible to shift from one to the other mode of recording without altering the control situation with regard to preload and preload. It should be noted that the two procedures implied differences in time courses of passive and dP/dt due to the non-linear length-tension relation of the resting muscle. For instance, during active stretch at constant dL/dt , there was a gradual increase in dP/dt whereas a stretch at constant dP/dt was associated with decreasing dL/dt (see further below).

Results

Fig. 1 shows original recordings of electrical and mechanical responses of the portal vein during passive stretch. The left panel is representative of the first type of experiment in which the muscle was subjected to changes in length at constant dL/dt and developed contractile force without active changes in length. The panel shows from top to bottom: output from



Effect of dynamic and static passive stretch on mechanical and extracellularly recorded electrical activity in the isolated rat portal vein. In the left panel the muscle was stretched by increasing the length by a constant rate. In the right panel the muscle was stretched to the same final length and passive force, was increased by increasing the load at constant rate.

transducer the muscle length (recorded by attaching the isotonic lever to the force sensor), the A.C. recording of the signals from the sucrose-gap electrodes, and the electronic spike counter output. In the control situation with a muscle length of 5.0 mm and a preload of 0.7 mN the preparation showed the usual pattern of phasic portal vein contractions associated with bursts of action potentials corresponding to an average of 28 spikes/min. Stretching the preparation by 40% at a rate of 2 mm/min (or about 0.7 per cent the initial length/s) led to a clear-cut increase in electrical and mechanical activity spike frequency increasing to 95 spikes/min during the dynamic stretch. Electrical activity in the following period of constant, increased length was only slightly enhanced (to 30 spikes/min) compared to control activity before stretch. The preparation was then returned to the original length and preload (not shown in the figure).

The right panel of Fig. 1 shows the results obtained in the same muscle under the second type of experimental conditions where the muscle shortened actively during the phasic contractions as shown in the length recording. Mean spike activity in the control period was now 27 spikes/min. The muscle was stretched to the same final length and passive force as in the left panel but in this case the stretch was applied by increasing the load on the lever at a constant dP/dt by means of the spiral spring. The continuous change in force could not be directly recorded under these conditions but was deduced from the calibration of the spring and is indicated at the top of the panel. It is evident that this type of passive stretch of the portal vein also led to a significant dynamic excitatory response, spike discharge increasing to 90 spikes/min despite the fact that the muscle could now counteract the passive lengthening by active shortening. At the later static stage of increased length and passive force, spike discharge was again only little changed compared to control activity.

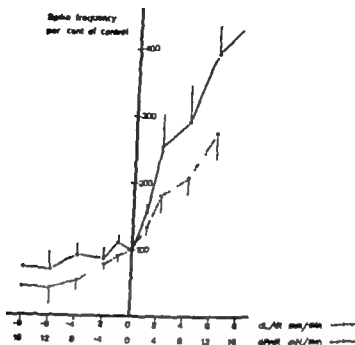
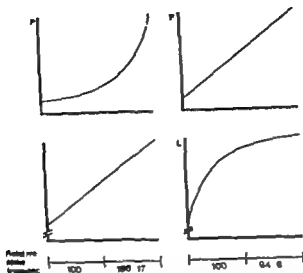


Fig. 2. Electrical response (mean \pm S.E.) of the isolated rat portal vein to dynamic changes in length and passive force at rates ranging from -8 to $+8$ mm/min or -16 to $+16$ mm/min, respectively. Spike activity at control length was set to 100. The values for dL/dt and dP/dt are the absolute rates of length change applied in the experiments; the figures for dP/dt are given as mean values since passive compliance varied between the different muscles.

The changes in spike discharge during the dynamic stretch shown in the left panel of Fig. 1 confirm the results of the earlier study (Johansson and Mellander 1975). The observations illustrated by the right panel of Fig. 1 indicate that passive stretch of the vascular smooth muscle is associated with a dynamic enhancement of spike activity also under conditions when active shortening can occur. Experiments were performed on a total of 7 portal vein preparations alternating repetitively throughout the individual experiment, between the two procedures for recording of mechanical responses and application of constant dL/dt and dP/dt , respectively. The total increase in length was always about 40 per cent of the muscle length prevailing at the low starting preload, but the rates of the changes in length and force were varied.

Fig. 2 summarizes the effects on spike activity obtained during the dynamic phases of stretch and shortening as a function of dL/dt or dP/dt in the two types of experimental procedure. The changes in spike activity are given as a percentage of the average frequency prevailing in the control period preceding each stretching of the muscle. Increases and decreases in spike activity with positive and negative dL/dt are qualitatively and quantitatively in agreement with the results reported in the earlier study. Fig. 2 shows that similar variations in spike discharge occurred when the muscles were passively stretched and shortened by application of positive and negative dP/dt under conditions where active shortening could take place. If anything, there is a relatively greater enhancement of spike discharge during stretch under these latter circumstances.



3 Schematic illustration showing how the rate of change in passive force increases when the stretch rate increased at constant rate (left diagrams of left panel of Fig. 1) and how the rate of change of length decreases but passive force is increased at constant rate (right diagrams of right panel of Fig. 1). At the bottom shows how the stretch period was divided into two halves. The spike activity in first half was set to 100. The number of spikes in the later half period was significantly greater when it was increasing during the stretch period (left diagrams) but not much changed when dP/dt is small and dL/dt decreasing during the stretch period (right diagrams).

As pointed out in the foregoing, the passive length-tension characteristics of the muscle implies that passive dP/dt will increase during a stretch at constant dL/dt , whereas dL/dt will decrease with time during a stretch at constant dP/dt . This can actually be seen in Fig. 1 and the schematic diagram in Fig. 3 may illustrate this point more clearly. In an attempt to differentiate between dL/dt and dP/dt as the adequate stimulus for the dynamic myogenic response, the following further analysis of the data was carried out. The different periods of dynamic stretch were divided into first and a second half (see Fig. 3), and the number of spikes occurring in each of these halves was compared. It was considered that if dL/dt were the important stimulating factor there should be an even distribution of spikes between the two half periods when stretch was produced by constant dL/dt (left diagrams of Fig. 3), whereas more spikes would occur in the first than in the second half during stretches at constant dP/dt (right diagrams of Fig. 3). Conversely if dP/dt represents the adequate stimulus, there should be a predominance of spike discharge in the second half period during stretches applied with constant dL/dt (left diagrams). It turned out that the number of spikes in the second half of the stretch periods at constant dP/dt was 94 ± 8 (mean ± 5 E.) per cent of the number obtained in the first half whereas in stretches at constant dL/dt , the figure for the second half was 166 ± 17 per cent of that for the first one. These findings indicate that stimulation of spike activity during stretch is more closely related to the change in passive force, dP/dt , than to the applied increase in length, dL/dt .

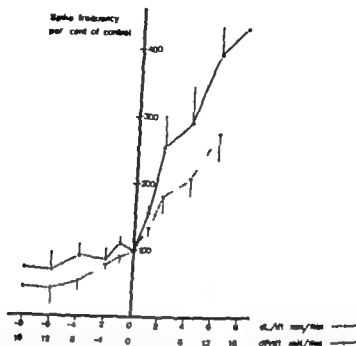
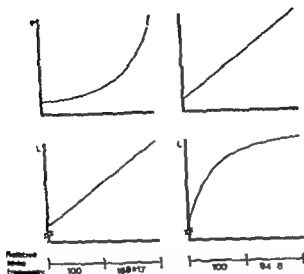


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3 Schematic illustration showing how the rate of change in passive force decreases when the stretch is increased at constant rate (left diagrams; cf. left panel of Fig. 2) and how the rate of change with time decreases when passive force is increased at constant rate (right diagrams; cf. right panel of Fig. 2). At the bottom is shown how the stretch period was divided into two halves. The spike activity in the first half was set to 100. The number of spikes in the later half period was significantly greater when it was increasing during the stretch period (left diagrams) but not much changed when dP/dt was fast and dL/dt decreasing during the stretch period (right diagrams).

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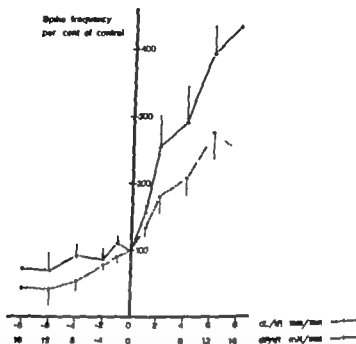


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The changes in spike discharge during the dynamic stretch shown in the left panel of Fig. 1 confirm the results of the earlier study (Johansson and Mellander 1975). The observations illustrated by the right panel of Fig. 1 indicate that passive stretch of the vascular smooth muscle is associated with a dynamic enhancement of spike activity also under conditions when active shortening can occur. Experiments were performed on a total of 7 portal vein preparations alternating repetitively throughout the individual experiment, between two procedures for recording of mechanical responses and application of constant force and dP/dt , respectively. The total increase in length was always about 40 per cent of the muscle length prevailing at the low starting preload, but the rates of the changes in length and force were varied.

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facilitate shortening in a cylindrical vessel while there is no equivalent to this mechanism in the present *in vitro* experiments. Therefore the results of the present study seem able with findings of myogenic autoregulation *in vivo* in response to both dynamic stimuli (Bæverfjord and Wehm 1969; Grünke *et al.* 1976). The further analysis of our results by Fig. 3 above indicated that the excitatory response to dynamic stretch are closely related to dP/dt than to dL/dt when considered for the preparation as a whole. The exact implication of this finding with respect to the nature of the sensing element localization in relation to the contractile units cannot be defined at present. It is of interest to note, however, that work on sensory mechanoreceptors have indicated that in some systems it is the ensuing change in tension rather than the actual length displacement which acts as the primary stimulus (Teorell 1971).

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Discussion

Autoregulatory maintenance of constant blood flow in response to variations in pressure is a more or less prominent characteristic of most vascular beds. The latter responsiveness requires, as an example, that a rise in arterial pressure elicits a corresponding increase in resistance to flow by vasoconstriction. The myogenic theory of autoregulation proposes that the transmural pressure itself creates the stimulus for enhancement of vascular smooth muscle tone. Since vessel caliber must become reduced in the actual example, it is evident that the sensing element involved in the myogenic autoregulatory reaction can be a simple length receptor responding to overall increases in vascular circumference. This kind of reasoning may apply also when the sensor responds to the rate of rise in pressure rather than to the level of elevated pressure.

In view of such considerations it was important to find out whether the dynamic response, demonstrated in isolated myogenically active vascular muscle (Johansson & Mellander 1975) would remain or whether it would become impaired if the muscle could counteract the applied stretch by active contractile shortening. Fig. 1 showed that the electrophysiological response to stretch was about the same when the muscle could shorten (right panel) as when contraction manifested itself purely as force (left panel). The diagram in Fig. 2 indicated that the relationships between spike frequency and rate of stretch or shortening are quite similar under the two conditions of mechanical response. It is evident therefore that active shortening neither abolished nor reduced the excitatory action of passive dynamic stretch.

The contractile responses which accompany the changes in spike discharge deserve special comments. The time average of contractile force during dynamic stretch (left panel of Fig. 1) increased as a result both of the enhancement of spike discharge and as a consequence of a shift to a more favourable section of the length-active tension curve as discussed in the earlier paper (Johansson and Mellander 1975). The enhancement of spike discharge and the increase in load will affect active contractile shortening in opposite directions. As indicated by the right panel of Fig. 1 the increased electrical activity during stretch acts to increase the time average of active shortening but this effect is curtailed by the increasing load which reduces the amplitude of the individual contractions. It appears from this recording that the excitatory response to stretch would be entirely insufficient to accomplish 'autoregulation' as the contractile shortening fails even to neutralize the passive lengthening. However, a number of important points should be considered in this context. First it should be realized that the relative magnitudes of passive lengthening and active shortening can be quite different under greater preloads than those applied here. In order to obtain sufficiently long periods of stretch over which measurements could be made it was necessary to start at a minimal preload in the present experiments, and in this situation the parallel elastic element of the muscle is very distensible so that passive lengthening becomes quite large. Active shortening fails to compensate for this lengthening despite the appreciable excitatory membrane response. Intact vascular beds also fail to autoregulate at low arterial pressures, i.e. in the low range of preloads on the vascular walls. The second point to be considered when comparing the right panel of Fig. 1 with the situation in the intact circulation is the *law of Laplace*

facilitate shortening in a cylindrical vessel while there is no equivalent to this mechanism in the present *in vitro* experiments. Therefore the results of the present study seem compatible with findings of myogenic autoregulation *in vivo* in response to both dynamic and static stimuli (Baez and Weiss 1969, Grönås *et al.* 1976). The further analysis of our results illustrated by Fig. 3 above indicated that the excitatory response to dynamic stretch is more closely related to dP/dt than to dL/dt when considered for the preparation as a whole. The exact implication of this finding with respect to the nature of the sensing element and its localization in relation to the contractile units cannot be defined at present. It is of interest to note, however, that work on sensory mechanoreceptors have indicated that in these systems it is the ensuing change in tension rather than the actual length displacement which acts as the primary stimulus (Teorell 1971).

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Discussion

Autoregulatory maintenance of constant blood flow in response to variations in pressure is a more or less prominent characteristic of most vascular beds. This kind of responsiveness requires, as an example, that a rise in arterial pressure elicits a corresponding increase in resistance to flow by vasoconstriction. The myogenic theory of autoregulation proposes that the transmural pressure itself creates the stimulus for enhancement of smooth muscle tone. Since vessel caliber must become reduced in the actual example it is evident that the sensing element involved in the myogenic autoregulatory reaction can be a simple length receptor responding to overall increases in vascular circumference. This kind of reasoning may apply also when the sensor responds to the rate of rise in pressure rather than to the level of elevated pressure.

In view of such considerations it was important to find out whether the dynamic responses, demonstrated in isolated myogenically active vascular muscle (Johansson & Mellander 1975) would remain or whether it would become impaired if the muscle could counteract the applied stretch by active contractile shortening. Fig. 1 showed that the electrophysiological response to stretch was about the same when the muscle could shorten (right panel) as when contraction manifested itself purely as force (left panel). The diagram in Fig. 2 indicated that the relationships between spike frequency and rate of stretch or shortening are quite similar under the two conditions of mechanical response. It is evident therefore that active shortening neither abolished nor reduced the excitatory action of passive dynamic stretch.

The contractile responses which accompany the changes in spike discharge deserve special comments. The time average of contractile force during dynamic stretch (left panel of Fig. 1) increased as a result both of the enhancement of spike discharge and as a consequence of the shift to a more favourable section of the length-active tension curve as discussed in the earlier paper (Johansson and Mellander 1975). The enhancement of spike discharge and the increase in load will affect active contractile shortening in opposite directions. As indicated by the right panel of Fig. 1 the increased electrical activity during stretch acts to increase the time average of active shortening but this effect is curtailed by the increasing load which reduces the amplitude of the individual contractions. It appears from this recording that the excitatory response to stretch would be entirely insufficient to accomplish 'autoregulation' as the contractile shortening fails even to neutralize the passive lengthening. However two important points should be considered in this context. First it should be realized that the relative magnitudes of passive lengthening and active shortening can be quite different at greater preloads than those applied here. In order to obtain sufficiently long periods of stretch over which measurements could be made it was necessary to start at a minimal preload in the present experiments, and in this situation the parallel elastic element of the muscle is very distensible so that passive lengthening becomes quite large. Active shortening fails to compensate for this lengthening despite the appreciable excitatory membrane response. Intact vascular beds also fail to autoregulate at low arterial pressures, i.e. in the low range of preloads on the vascular walls. The second point to be considered when comparing the right panel of Fig. 1 with the situation in the intact circulation is the ~~the~~ law of Laplace

smooth muscle cells (Kosterlitz and Watt 1965, Oberdorf and Kronberg 1969). It has been noted that stimulation of α -adrenoceptors inhibits the release of acetylcholine from guinea ileum and rabbit jejunum (Paton and Vizi 1969, Kosterlitz *et al.* 1970, Vizi and Knoll 1971). Gershon (1967) failed to find any effect of acetylcholine release on the stimulation of sympathetic nerves in the rabbit jejunum, although the preparation was relaxed. He did state, however, whether the relaxation was mediated by α - or β -adrenoceptors. On electrophysiological recording of spontaneously active neurons in Auerbach's plexus of the guinea pig, α -adrenoceptor stimulation was found to inhibit the spike activity (Sato *et al.* 1973).

The adrenergic innervation of the gastrointestinal tract has recently been reviewed by Nisä and Costa (1974). There is both histochemical and electronmicroscopic evidence of the adrenergic innervation of intramural ganglion cells in the myenteric plexus of several mammalian species (Norberg 1964, Jacobowitz 1965, Åberg and Erenkö 1967, Gabella 1972a). In contrast, a virtual absence of adrenergic innervation has been found in the enteric longitudinal muscle layer of the small intestine of the monkey, rat, cat, guinea pig and rabbit (Norberg 1964, Jacobowitz 1965, Costa and Gabella 1971, Silva *et al.* 1971). Some portions of the gastrointestinal tract receive direct adrenergic innervation into the smooth muscle, such as the circular muscle of the small and large intestines and the taenia coli of the rat, guinea pig and rabbit (Åberg and Erenkö 1967, Furness 1970, Costa and Gabella 1971).

The findings summarized above suggest that α -receptor induced relaxation of the intestine depends on an action on the intramural cholinergic neurons and/or on its nerve endings. They do not exclude the possibility, however, that inhibitory α -adrenoceptors located in the smooth muscle cells may contribute to the relaxation. Several investigators have reported that relaxation mediated by α -adrenoceptors has been resistant to blockade by atropine or hexamethonium (Lee 1970, Bowman and Hall 1970). Lee (1970) observed that after blockade of β -adrenoceptors, epinephrine was more potent in relaxing guinea pig circular ileum contracted by histamine, in the absence than in the presence of atropine. He therefore suggested that the organ was relaxed by the inhibition of α -adrenoceptors located both in smooth muscle cells and in cholinergic neurons.

When investigating the relation between biochemical and biophysical events following stimulation of an adrenergic receptor it is of the utmost importance for interpretation of the observations that only one type of receptor is stimulated at a time. This approach was used in differentiating between the metabolic effects induced by α - and β -adrenoceptors in rabbit colon (Anderson and Mohme Lundholm 1970, Anderson 1972). The possibility that α -adrenoceptors may be located both in neurons and in smooth muscle cells in the intestine has to be considered when investigating the biochemical and biophysical events following stimulation of these receptors in the guinea pig and rabbit intestines.

When studying relaxing mechanisms having neuronal or muscular point of action there are some theoretical and methodological problems which have to be taken into account. A muscle contracted as a result of activation of neural elements in the intestine may be relaxed by an inhibitory mechanism located either in the neurons or in the smooth muscle cells of the organ. If the preparation is contracted by direct activation of the smooth muscle cells,

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The localization of adrenergic receptors mediating a relaxing action was investigated in innervated and denervated longitudinal muscle strips from guinea pig ileum and rabbit jejunum. Denervated preparations were contracted by drugs that had a direct effect on smooth muscle cells, such as acetylcholine and histamine, but not by stimuli acting on cholinergic neurons, such as electrical field stimulation or neostigmine. After blockade of β -adrenoceptors, norepinephrine relaxed the innervated guinea pig ileum contracted by electrical field stimulation, by stimulating α -adrenoceptors. Norepinephrine in low concentrations did not relax denervated preparations contracted by agents acting directly on smooth muscle. In high concentrations, it relaxed denervated preparations by a nonadrenergic mechanism, resistant to α - and β -receptor blockade, but which was also activated by 1-(3,4-dihydroxyphenyl) ethanol. Phenylephrine only had a weak agonistic effect on the electrically stimulated innervated preparation and did not relax the denervated preparation. The denervated rabbit intestine contracted by acetylcholine was relaxed by norepinephrine and phenylephrine by stimulation of α -adrenoceptors. In the innervated preparations both drugs were more effective. Inhibiting contractions induced by electrical field stimulation of intestine that those induced by exogenous acetylcholine. Both the denervated guinea pig and rabbit intestine were relaxed by stimulation of β -adrenoceptors. It is suggested that in the guinea pig ileum α -adrenoceptors mediating relaxation are located in cholinergic neurons, whereas in rabbit jejunum they are located both in these neurons and in the smooth muscle cells. β -Adrenoceptors are located in the smooth muscle cells of both organs.

The relaxing action of catecholamines in the intestine of many mammalian species is mediated by adrenergic α - and β -receptors (Ahlfqvist and Levy 1959, Furchgott 1960, Bucknell and Whitney 1964, Andersson and Mohme Lundholm 1969, Bowman and Hargrett-Nelson 1970). The biochemical and biophysical events associated with the stimulation of these receptors differ in many respects (Jenkinson and Morton 1967, Brody and Diamond 1968, Bülbirg and Tomita 1969, Andersson and Mohme Lundholm 1970, Andersson and Bülbirg 1971). It has been suggested that the relaxing effect mediated by α -adrenoceptors in guinea pig longitudinal ileum is due to an inhibition of cholinergic neurons innervating the intestine while the β -adrenoceptor-mediated effect is elicited by a direct action on

smooth muscle cells (Kosterlitz and Watt 1965, Oberdorf and Kronberg 1969). It has been found that stimulation of α -adrenoceptors inhibits the release of acetylcholine from guinea pig and rabbit jejunum (Paton and Vizi 1969). Kosterlitz *et al.* 1970, Vizi and Knoll-Jershon (1967) failed to find any effect of acetylcholine release on the stimulation of autonomic nerves in the rabbit jejunum, although the preparation was relaxed. He did not know, however, whether the relaxation was mediated by α - or β -adrenoceptors. On microelectrophysiological recording of spontaneously active neurons in Auerbach's plexus of the pig, α -adrenoceptor stimulation was found to inhibit the spike activity (Sato *et al.*

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The findings summarized above suggest that α -receptor induced relaxation of the intestine acts on an action on the intramural cholinergic neurons and/or on its nerve endings. This does not exclude the possibility, however, that inhibitory α -adrenoceptors located in the smooth muscle cells may contribute to the relaxation. Several investigators have reported that relaxation mediated by β -adrenoceptors has been resistant to blockade by atropine or tetrodotoxin (Lee 1970, Bowman and Hall 1970). Lee (1970) observed that after blockade of β -adrenoceptors, epinephrine was more potent, in relaxing guinea pig circular ileum contracted by histamine, in the absence than in the presence of atropine. He therefore concluded that the organ was relaxed by the inhibition of α -adrenoceptors located both in smooth muscle cells and in cholinergic neurons.

When investigating the relation between biochemical and biophysical events following stimulation of an adrenergic receptor it is of the utmost importance for interpretation of the results that only one type of receptor is stimulated at a time. This approach was used in differentiating between the metabolic effects induced by α - and β -adrenoceptors in rabbit jejunum (Andersson and Mölne-Lundholm 1970, Andersson 1972). The possibility that α -adrenoceptors may be located both in neurons and in smooth muscle cells in the intestine has to be considered when investigating the biochemical and biophysical events following stimulation of these receptors in the guinea pig and rabbit intestines.

When studying relaxing mechanisms having neuronal or muscular point of action there are some theoretical and methodological problems which have to be taken into account. A smooth muscle contracted as a result of activation of neural elements in the intestine may be relaxed by an inhibitory mechanism located either in the neurons or in the smooth muscle cells of the organ. If the preparation is contracted by direct activation of the smooth muscle cells,

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or pulses at 0.1–30 Hz either continuously or for 10 s at intervals of one min with Grass SD9 stimulator and bones contracted (max). The strength of the stimulating field was always experimental. Specimens were kept in Krebs solution of the following composition (in mM): NaCl 122.0, KCl 4.0, CaCl_2 1.0, CaCl_2 2.5, NaHCO_3 15.4, KH_2PO_4 1.2 and glucose 5.5, equilibrated with gas mixture of CO_2 and 95% O_2 . The temperature of the Krebs solution was 37°C. The preparations were left in the solution for 1 h before the experiments were started.

Blue blue stained preparations

Intact or denervated preparations were supravitaly stained in methylene blue at 37°C. The staining solution was composed of 0.3 g methylene blue and 9.0 g NaCl in 1 000 ml of water. The preparations were placed in Krebs solution and mounted on object glasses, and photographs were obtained immediately (Leitz Elmer (f=5 cm, f=3.5)). Some of the preparations were fixed in 8% $(\text{NH}_4)_2\text{MoO}_4$, dehydrated in ethanol and embedded in methyl methacrylate. Sections 10 μm thick were cut for microscopical examination.

Statistical methods

Response curves were treated statistically. Each of them was fitted into the logistic function

$$E = M \frac{A}{A + E^p}$$

applying Taylor's theorem for non-linear least square regression analysis (Parter and Wood 1971). In the formula, A is the concentration, E the ED_{50} value and M the maximal response to the drug. p is a constant which depends on the slope of the dose-response curve. Values obtained experimentally are given as the arithmetic mean \pm S.E. Differences were tested by Student's t -test for paired observations. For testing significance of ED_{50} values their logarithmic transformation ($\log D_5$) was used.

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Following were used: Acetylcholine chloride (Sigma), magnesium oxide (Ciba), atropine sulphate (CO), dibenzamine hydrochloride (Astra), eserine sulphate (Merck), histamine chloride (ACO), nifedipine (Damon), dl -isoprenaline sulphate (WB), carbacholone chloride (ACO), morphine chloride (CO), l -isoprenaline bitartrate (Sigma), oxymetazoline chloride (Draco), pseudoephedrine bitartrate (Astra Rabis), l -phenylephrine hydrochloride (Sigma), phenylephrine methanesulphonate (Ciba), promethazine chloride (ICI), atrolol hydrochloride (Mead-Johnson), tolazoline hydrochloride (Ciba-Geigy), tolazoline (Sigma).

Results

Intestinal strips from guinea pig ileum

Histological examination of methylene blue stained preparations. The innervated preparations showed a dense network of dark-blue stained nervous elements (Fig. 1 A). In the microscope ganglia with several ganglionic cells were seen lying on the surface of the smooth muscle. Interconnecting nerve bundles were sometimes present between the ganglia. When the longitudinal muscle was denervated, as described under Methods (p. 192) the proximal part usually contained the nervous plexus and often some circular muscle fibres. In the rest of the preparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal parts became completely free from nervous elements (Fig. 1 B). In the embedded denervated preparations no nervous elements at all could be identified.

Contractile responses of innervated and denervated preparations. The innervated strips were contracted by acetylcholine and histamine which probably acted both on neural and muscular receptors. The preparations were also contracted by eserine and nicotine. The contractile effect of nicotine was inhibited by atropine (3.5×10^{-6} M) (Fig. 2 A, 1.5). In the

with no neural participation, the muscle can only be relaxed by a direct effect on cells. There are few drugs, if any, that contract intestinal smooth muscle selectively on its neurons. Drugs such as acetylcholine, angiotensin, serotonin and ions such as barium and potassium, contract guinea pig ileum and other preparations by stimulating both cholinergic neurons and muscle cells (Ambache 1955; Paton and Vane 1963; Paton and Zar 1968). This problem may be overcome by a drug such as atropine to block the cholinergic receptors. Such an approach has advantages, however. Firstly, the introduction of other drugs increases the risk of unknown effects, e.g. interference with the receptor mechanism under investigation. Secondly, the presence of an atropine-resistant, non-cholinergic excitatory neural input in guinea pig ileum has been suggested (Ambache and Freeman 1968). In the main approach in the attempt to differentiate between α -adrenoceptors located on nerves and those located at muscle cells has been to compare the responses of innervated and denervated preparations.

Methods

Male guinea pigs and rabbits of either sex were killed by a blow on the neck. The small intestine was removed, the parts adjacent to the pylorus and colon being discarded, and dissected free from the mesentery. The longitudinal muscle layers were removed from the circular muscle by different techniques either innervated or denervated longitudinal muscle strips.

Innervated preparations

Guinea pig. The innervated specimens from the guinea pig were prepared as described by Paton (1968). A piece of the intestine obtained approximately 10 cm proximal to the ileocecal sphincter was pinned to a glass rod. The longitudinal muscle was removed from one end by stroking tangentially from the border with a wisp of cotton wool. The rest of the muscle was then stripped off by pulling from the free end.

Rabbit. Innervated specimens from the rabbit were prepared as described by Ambache (1955) but the jejunal part of the intestine was chosen instead of the ileum.

Denervated preparations

Guinea pig. To obtain denervated preparations from the guinea pig the method described by Paton (1968) was used. A longer piece of the intestine was threaded on the glass rod and the longitudinal muscle was dissected free from one end as described above. A long piece of longitudinal muscle was then prepared. In successful preparations about 25–30 cm of the ileum could be prepared in this way without breaking up into thin strands. Under a stereo microscope Auerbach's plexus is readily identifiable by staining. It is often found that the distal parts of the preparation contain the plexus but that this plexus disappears in wedge-shaped areas towards the proximal end so that this end becomes completely plexus-free. A plexus-free part was selected and cut away from the plexus-retaining portion.

Rabbit. For denervating rabbit jejunum essentially the same technique was used as for guinea pig. A long piece of longitudinal muscle was obtained and inspected under the stereo microscope. The wedge-shaped disappearance of the plexus seen in the guinea pig ileum was not observed in rabbit intestine. Instead, plexus-free parts were often surrounded by innervated areas, or in some places innervated parts were left inside areas of denervated muscle. The innervated and denervated parts were clearly identifiable without staining and the denervated piece was carefully cut out with fine pair of scissors.

The preparations were mounted in special holders, described by Lundholm and Mohmed (1966) and immersed in organ baths with a capacity of 70 ml. The tension of the preparations was recorded isometrically by means of a FT03 transducer and Grass polygraph. Electrically stimulated preparations were mounted in a water-jacketed 4 ml perspex chamber. The walls of the chamber 1.0–2.0 cm in diameter were mounted 1 cm apart parallel to the interposed preparation, permitting a current to be passed through the Krebs solution and the muscle. Stimulation was controlled by a Grass S4 stimulator.

pulses at 0.1–50 Hz either continuously or for 10 s at intervals of one min with Grass SD9 stimulator whose contracted tissue. The strength of the stimulating field was always supramaximal. Experiments were kept in Krebs solution of the following composition (in mM): NaCl 122.0, KCl 3.6, CaCl_2 2.5, NaHCO_3 15.4, KH_2PO_4 1.2 and glucose 5.5 equilibrated with gas mixture of 95% O_2 . The temperature of the Krebs solution was 37°C. The preparations were left in the solution for 1 h before the experiments were started.

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Dose-response curves were treated statistically. Each of them was fitted into the logistic function

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using Taylor's theorem for non-linear least square regression analysis (Parker and Wexel 1971) formula, A the concentration, K the ED_{50} value and M the maximal response to the drug. p is an index which is dependent on the slope of the dose-response curve. Values obtained experimentally are in the arithmetic mean \pm S.E. Differences were tested by Student's t -test for paired observations. Testing significance of ED_{50} values their logarithmic transformation (pED_{50}) was used.

Drugs used: Acetylcholine chloride (Sigma), angorestin amide (Ciba), atropine sulphate (H. Hoescht), dibenzylamine hydrochloride (Astra), ecarine suberylure (Merck), histamine chloride (ACO), isodrine (Domec), α -1-norpropranolol sulphate (W.B.), carbachol chloride (ACO), morphine chloride (H. Hoescht), 1-norpropranolol butyrate (Sigma), oxytocin chloride (Draco), pentolinium butyrate (H. Hoescht), 1-phenylephrine hydrochloride (Sigma), phentolamine methanesulphonate (Ciba), procaine chloride (ICI), sotalol hydrochloride (Mead-Johnson), cotinine hydrochloride (Ciba-Geigy), detomidine (Sigma).

Results

Intervated strips from guinea pig ileum

Microscopical examination of methylene blue stained preparations. The innervated preparations showed a dense network of dark-blue stained nervous elements (Fig. 1A). In the microscope field with several ganglionic cells were seen lying on the surface of the smooth muscle. Interconnecting nerve bundles were sometimes present between the ganglia. When the longitudinal muscle was denervated, as described under Methods (p. 192), the proximal part usually contained the nervous plexus and often some circular muscle fibres. In the rest of the preparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal part became completely free from nervous elements (Fig. 1B). In the embedded denervated preparations no nervous elements at all could be identified.

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Guinea pig. To obtain denervated preparations from the guinea pig the method described by Paton (1968) was used. A longer piece of the intestine was threaded on the glass rod and the longitudinal muscle was dissected free at one end as described above. A long piece of longitudinal muscle was then prepared by breaking up into thin strands. Under a stereo microscope Auerbach's plexus is readily identifiable by staining. It is often found that the distal parts of the preparation contain the plexus but that the plexus disappears in a wedge towards the proximal end so that this end becomes completely plexus-free part was selected and cut away from the plexus-retaining portion.

Rabbit. For denervating rabbit jejunum essentially the same technique was used as for guinea pig. A long piece of longitudinal muscle was obtained and inspected under the stereo microscope. The wedge-shaped disappearance of the plexus seen in the guinea pig ileum was not observed in the rabbit intestine. Instead plexus-free parts were often surrounded by innervated areas, or in some places by innervated parts were left inside areas of denervated muscle. The innervated and denervated areas were clearly identifiable without staining and the denervated piece was carefully cut out with a fine pair of forceps.

The preparations were mounted in special holders, described by Lundholm and Molane-Lundholm (1966) and immersed in organ baths with a capacity of 20 ml. The tension of the preparations was measured isometrically by means of FT03 transducer and Grass polygraph. Electricity stimulated preparations were mounted in a water-jacketed 4 ml perspex chamber. The walls of the chamber (2 cm long) were mounted 1 cm apart parallel to the interposed preparation, permitting an electric current to be passed through the Krebs solution and the muscle. Stimulation was produced by 1

pulses at 0.1–50 Hz either continuously or for 10 s at intervals of one min with Grass SD9 stimulator and home constructed timer. The strength of the stimulating field was always supramaximal. Specimens were kept in Krebs solution of the following composition (in mM): NaCl 122.0, KCl 4.0, CaCl_2 1.2, CaCl_2 5, NaHCO_3 19.4, KH_2PO_4 1.2 and glucose 5.5, equilibrated with gas mixture of O_2 and 95% CO_2 . The temperature of the Krebs solution was 37°C. The preparations were left in the bath for 1 h before the experiments were started.

Low blue stained preparations

Intact or denervated preparations were supravitaly stained in methylene blue at 37°C. The staining solution consisted of 0.3 g methylene blue and 9.0 g NaCl in 1 000 ml of water. The preparations were fixed in Krebs solution and mounted on object glasses, and photographs were obtained immediately (Leitz Elumar II 5 cm, f 3.5). Some of the preparations were fixed in 8% $(\text{NH}_4)_2\text{MoO}_4$, dehydrated in ethanol and embedded in methyl methacrylate. Sections 10 μm thick were cut for microscopy examination.

Data analysis

Dose-response curves were treated statistically. Each of these was fitted into the logistic function

$$E = M \frac{A^P}{A^P + K^P}$$

applying Taylor's theorem for non-linear least square regression analysis (Parker and Ward 1971). In formula, A is the concentration, E the ED_{50} value and M the maximal response to the drug. P is assumed to be independent of the slope of the dose-response curve. Values obtained experimentally are given as the arithmetic mean \pm S.E. Differences were tested by Student's t -test for paired observations or using significance of ED_{50} values their logarithmic transformation (pED_{50}) was used.

Drugs

Following are used: Acetylcholine chloride (Regina), ergometrine maleate (Ciba), atropine sulphate (Dr. Williams), 4-aminocyanine hydrochloride (Astra), eserine salicylate (Merck), histamine chloride (ACO), indolamine (Dr. Williams), di-nopropionyl sulphate (WB), carbachol chloride (ACO), morphine chloride (ACO), 1-norepinephrine bitartrate (Regina), atyranisole chloride (Draco), pentolinium bitartrate (Kabi), 1-phenylephrine hydrochloride (Regina), phenolamine methanesulphonate (Ciba), promethazine chloride (ICI), tolazoline hydrochloride (Mead-Johnson), tolazoline hydrochloride (Ciba-Geigy), nifedipine (Regina).

Results

Longitudinal strips from guinea pig ileum

Histological examination of methylene blue stained preparations. The innervated preparations showed a dense network of dark-blue stained nervous elements (Fig. 1 A). In the microscope fields with several ganglionic cells were seen lying on the surface of the smooth muscle. Interconnecting nerve bundles were sometimes present between the ganglia. When the longitudinal muscle was denervated, as described under Methods (p. 192), the proximal parts usually contained the nervous plexus and often some circular muscle fibres. In the rest of the preparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal parts became completely free from nervous elements (Fig. 1 B). In the embedded denervated preparations no nervous elements at all could be identified.

Contractile responses of innervated and denervated preparations. The innervated strips are contracted by acetylcholine and histamine which probably acted both on neural and muscular receptors. The preparations were also contracted by eserine and nicotine. The contractile effect of nicotine was inhibited by atropine ($3.5 \cdot 10^{-6}$ M) (Fig. 2 A, 1.5). In the

with no neural participation, the muscle can only be relaxed by a direct effect on cells. There are few drugs, if any, that contract intestinal smooth muscle selectively without any effect on its neurons. Drugs such as acetylcholine, angiotensin, serotonin and ions such as barium and potassium, contract guinea pig ileum and other intestinal preparations by stimulating both cholinergic neurons and muscle cells (Ambache and Li 1955, Paton and Vane 1963, Paton and Zar 1968). This problem may be overcome by using a drug such as atropine to block the cholinergic receptors. Such an approach has its advantages, however. Firstly the introduction of other drugs increases the risk of producing unknown effects, e.g. interference with the receptor mechanism under investigation. Secondly the presence of an atropine-resistant non-cholinergic excitatory neural mechanism in guinea pig ileum has been suggested (Ambache and Freeman 1968). In this work the main approach in the attempt to differentiate between α -adrenoceptors located at nerve and those located at muscle cells has been to compare the responses of innervated and denervated preparations.

Methods

Male guinea pigs and rabbits of either sex were killed by a blow on the neck. The small intestine was removed, the parts adjacent to the pylorus and colon being discarded, and dissected free from the mesentery. The longitudinal muscle layers were removed from the circular muscle by different techniques to give either innervated or denervated longitudinal muscle strips.

Innervated preparations

Guinea pig. The innervated specimens from the guinea pig were prepared as described by Ruzs (1968). A piece of the intestine obtained approximately 10 cm proximal to the ileocaecal sphincter was dissected free at one end by stroking tangentially from the mesenteric border with a wisp of cotton wool. The rest of the muscle was then stripped off by pulling gently to the free end.

Rabbit. Innervated specimens from the rabbit were prepared as described by Ambache (1954), but the jejunal part of the intestine was chosen instead of the ileum.

Denervated preparations

Guinea pig. The obtuse denervated preparations from the guinea pig by the method described by Paton (1968) was used. A longer piece of the intestine was threaded on the glass rod and the longitudinal muscle was dissected free at one end as described above. A long piece of longitudinal muscle was then pulled out. In successful preparations about 25–30 cm of the ileum could be prepared in this way. Instead of breaking up into thin strands, under a stereo microscope Auerbach's plexus is readily identified by staining. It is often found that the distal parts of the preparation contain the plexus but that the plexus disappears in a wedge towards the proximal end so that this end becomes completely plexus-free. This plexus-free part was selected and cut away from the plexus-retaining portion.

Rabbit. For denervating rabbit jejunum essentially the same technique was used as for guinea pig. A long piece of longitudinal muscle was obtained and inspected under the stereo microscope. The wedge-shaped disappearance of the plexus seen in the guinea pig ileum was not observed in the rabbit intestine. Instead, plexus-free parts were often surrounded by innervated areas, or in some places by innervated parts were left inside areas of denervated muscle. The innervated and denervated areas were clearly identifiable without staining and a denervated piece was carefully cut out with a fine pair of scissors.

The preparations were mounted in special holders, described by Lundholm and Molne-Lundholm (1966) and immersed in organ baths with a capacity of 20 ml. The tension of the preparations was recorded isometrically by means of a FT03 transducer and a Grass polygraph. Electrically stimulated preparations were mounted in a water-jacketed 4 ml perspex chamber. In the walls of the chamber two 1 cm long platinum electrodes were mounted 1 cm apart parallel to the interposed preparation, permitting an electric current to be passed through the Krebs solution and the muscle. Stimulation was produced by a

jects at 0.1–50 Hz either continuously or for 10 s at intervals of one min with Grass SD9 stimulator and Grass constructed timer. The strength of the stimulating field was always supramaximal. Preparations were kept in a Krebs solution of the following composition (mM): NaCl 122.0, KCl 5.4, CaCl₂ 2.5, NaHCO₃ 15.4, KH₂PO₄ 1.2 and glucose 5.5, equilibrated with a gas mixture of 95% O₂. The temperature of the Krebs solution was 37°C. The preparations were left in the bath for 1 h before the experiments were started.

in blue stained preparations

Intervated preparations were supravitaly stained in methylene blue at 37°C. The staining solution consisted of 113 g methylene blue and 9.0 g NaCl in 1000 ml of water. The preparations were kept in Krebs solution and mounted on object glasses, and photographs were obtained immediately with Elmer (F 5 cm, 1:3.5). Some of the preparations were fixed in 8% (NH₄)₂MoO₄, dehydrated in alcohol and ethanol and embedded in methyl methacrylate. Sections 10 µm thick were cut for microscopical examination.

data analysis

Dose-response curves were treated statistically. Each of them was fitted into the logistic function

$$E \sim M \frac{A^P}{A^P + K^P}$$

where Taylor's theorem for non-linear least square regression analysis (Parker and Ward 1971). In this formula, A is the concentration, K the ED₅₀ value and M the maximal response to the drug. P is an index dependent on the slope of the dose-response curve. Values obtained experimentally are in the arithmetic means \pm S.E. Differences were tested by Student's t -test for paired observations. Testing significance of ED₅₀ values their logarithmic transformation (pED_{50}) was used.

Drugs used: Acetylcholine chloride (Sigma), angiotensin amide (Ciba), atropine sulphate (A), dibenzylamine hydrochloride (Astra), ecarine sulphate (Merck), histamine chloride (ACO), isodrenaline (Dumex), 41-isoprenaline sulphate (WB), carbachol chloride (ACO), morphine chloride (A), 1-norphenylphrine bitartrate (Sigma), oxymetazoline chloride (Draco), pentolamine bitartrate (A), 1-phenylephrine hydrochloride (Sigma), phentolamine methanesulphonate (Ciba), propanolol chloride (ICI), sotalol hydrochloride (Mead-Johnson), tolazoline hydrochloride (Ciba-Geigy), doxamine (Sigma).

Results

Intervated strips from guinea pig ileum

Intervated examination of methylene blue stained preparations. The intervated preparations had a dense network of dark-blue stained nervous elements (Fig. 1A). In the microscope light all several ganglionic cells were seen lying on the surface of the smooth muscle. Interconnecting nerve bundles were sometimes present between the ganglia. When the longitudinal muscle was deservated, as described under Methods (p. 192) the proximal parts still contained the nervous plexus and often some circular muscle fibres. In the rest of the preparation the nervous plexus disappeared in a wedge-shaped fashion so that the distal parts became completely free from nervous elements (Fig. 1B). In the embedded deservated preparations no nervous elements at all could be identified.

Contractile responses of intervated and deservated preparations. The intervated strips were contracted by acetylcholine and histamine which probably acted both on neural and muscular receptors. The preparations were also contracted by ecarine and nicotine. The contractile effect of nicotine was inhibited by atropine ($3.5 \cdot 10^{-6}$ M) (Fig. 2A, 1–5). In the



Fig. 1 (A) Photomicrograph of an innervated strip of guinea pig ileum stained with methylene blue. A plexus is seen dark-stained against the paler smooth muscle. At the margins of the preparation plexus has been removed so that these parts appear plexus-free. Horizontal bar indicates 1 mm. (B) Denervated strip of guinea pig ileum stained with methylene blue. 'T' (the left, at the proximal end, a small mass of nervous tissue is seen. In the rest of the preparation the longitudinal muscle is free from innervation and ganglia.

denervated strips histamine and acetylcholine elicited a contractile effect but neither nicotine did not (Fig. 2 B, 1-4)

Electrical field stimulation by (1 ms) single pulses at 0.1 Hz induced a twitch contraction at each pulse in the innervated preparations. The contractions were completely abolished by atropine (3.5×10^{-6} M) and by tetrodotoxin (3×10^{-6} M) (Fig. 3 A 1-3). Tetanic field stimulation (10 Hz for 10 s) produced stronger contractions which were reduced but

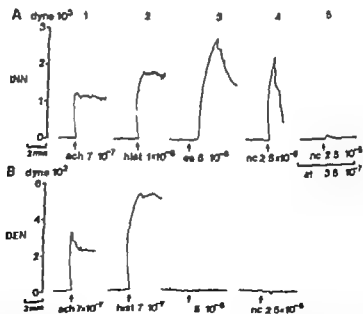
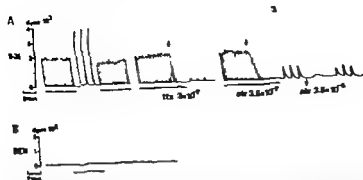


Fig. 2 (A) Responses of an innervated strip of guinea pig ileum A 1-4 Acetylcholine 7×10^{-7} M (ach), histamine 1×10^{-6} M (hist), serine 5×10^{-6} M (es) and nicotine 2.5×10^{-6} M (nc), respectively. A. Nicotine 2.5×10^{-6} M (nc) in the presence of atropine 3.5×10^{-6} M (at) which had been added 10^{-2} previously. (B) Responses of a denervated strip of guinea pig ileum B 1-4 Acetylcholine 7×10^{-7} M (ach), histamine 7×10^{-7} M (hist), serine 5×10^{-6} M (es) and nicotine 2.5×10^{-6} M (nc), respectively.



3 Responses of an innervated strip of guinea pig ileum. A 1-3 The horizontal bars indicate field stimulation at 0.1 Hz with 1 ms rectangular pulses. At the dots, field stimulation at 10 Hz. The arrows indicate addition of tetrodotoxin $3 \cdot 10^{-6}$ M (TTX) and atropine $3.5 \cdot 10^{-6}$ - $3.5 \cdot 10^{-5}$ M (ATR), respectively. B Responses of a denervated strip of guinea pig ileum. (These preparations were stimulated as in experiment 2 B, 1-4). The horizontal bar indicates electrical field stimulation at 0.1 Hz with 1 ms pulses. At the dots, field stimulation at 10 Hz for 10 s. At the asterisks, field stimulation at 50 Hz for 10 s.

ed by atropine ($3.5 \cdot 10^{-6}$ - $3.5 \cdot 10^{-5}$ M). Tetrodotoxin almost completely inhibited the response (Fig. 3 A, 1-3). In the denervated preparations both kinds of electrical stimulation were totally ineffective (Fig. 3 B). These observations indicate that on electrical stimulation an innervated preparation was contracted by a cholinergic mechanism. At higher stimulation rates a non-cholinergic mechanism might also have contributed to the contraction.

α -adrenoceptor mediated relaxation in innervated strips. In the innervated strip in β -adrenoceptors were blocked by alprenolol ($5 \cdot 10^{-6}$ M), norepinephrine ($3 \cdot 10^{-6}$ M) led to the contractions elicited by electrical stimulation at 0.1 Hz to $92 \pm 5\%$ (n = 6) (Fig. 4 A, 1). This effect was almost completely blocked by tolazoline ($3 \cdot 10^{-4}$ M) (Fig. 4 A, 2). When the innervated strip was contracted by acetylcholine ($7 \cdot 10^{-4}$ M), the same contraction of norepinephrine ($3 \cdot 10^{-6}$ M) relaxed the preparation to $52 \pm 2\%$ (n = 5) (Fig. 4 A, 3). Phenylephrine ($1 \cdot 10^{-6}$ M) produced only minor and often transitory effects in the electrically stimulated preparations (Fig. 4 A, 3). Following contraction by acetylcholine, epinephrine in concentrations of up to $5 \cdot 10^{-6}$ M had a weak transitory relaxing action (Fig. 4 B, 2) or none at all.

Relaxant response in denervated strip. To ascertain that the preparations were functionally intact and that their contractile ability was not damaged, they were always tested with nicotine ($5 \cdot 10^{-6}$ M) and acetylcholine ($7 \cdot 10^{-4}$ M). The mean tension induced by nicotine in denervated preparations was only 1.6 ± 0.7 g (n = 9) of that induced by acetylcholine in the same strip. In the innervated strips, in contrast, the nicotine contraction was 231.9 ± 10.5 g (n = 5) of the mean tension caused by acetylcholine. When tested, the denervated preparations invariably failed to respond to nicotine or tetanic field stimulation.

When the relaxing action of α -adrenoceptor-stimulation was tested in the denervated strips, these were pre-treated with alprenolol ($5 \cdot 10^{-6}$ M) to block β -adrenoceptors and then contracted by acetylcholine ($7 \cdot 10^{-4}$ M), carbachol ($7 \cdot 10^{-6}$ M), or histamine ($1 \cdot 10^{-4}$ M). Norepinephrine in a concentration of $3 \cdot 10^{-6}$ M, which relaxed the innervated strip, had



Fig. 1 (A) Photomicrograph of an innervated strip of guinea pig ileum stained with methylene blue. The plexus is seen dark-stained against the paler smooth muscle. At the margins of the preparation plexus has been removed so that these parts appear plexus-free. Horizontal bar indicates 1 mm. (B) Denervated strip of guinea pig ileum stained with methylene blue. To the left, at the proximal end, a small mass of nervous tissue is seen. In the rest of the preparation the longitudinal muscle is free from nerve and ganglia.

denervated strips histamine and acetylcholine elicited a contractile effect but cereulonic nicotine did not (Fig. 2 B, 1-4)

Electrical field stimulation by (1 ms) single pulses at 0.1 Hz induced a twitch contraction at each pulse in the innervated preparations. The contractions were completely abolished by atropine (3.5×10^{-6} M) and by tetrodotoxin (3×10^{-6} M) (Fig. 3 A, 1-3). Tetanic field stimulation (10 Hz for 10 s) produced stronger contractions which were reduced but

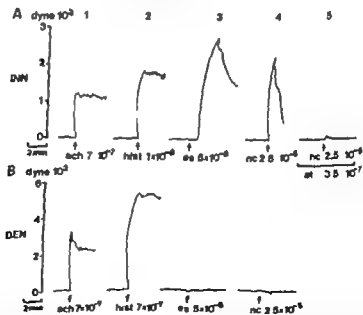
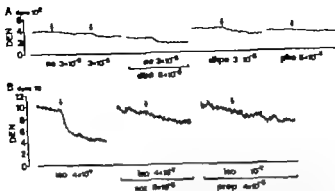


Fig. 2. (A) Responses of an innervated strip of guinea pig ileum. A 1-4 Acetylcholine 7×10^{-7} M (ach), histamine 1×10^{-6} M (hist), eserine 5×10^{-6} M (es) and nicotine 2.5×10^{-6} M (nc), respectively. A 1-4 Nicotine 2.5×10^{-6} M (nc) in the presence of atropine 3.5×10^{-6} M (atr) which had been added 10 s previously. (B) Responses of a denervated strip of guinea pig ileum. B 1-4 Acetylcholine 7×10^{-7} M (ach), histamine 1×10^{-6} M (hist), eserine 5×10^{-6} M (es) and nicotine 2.5×10^{-6} M (nc), respectively.



5. (A) Responses of denervated strips of guinea pig ileum. Contraction of the preparations was maintained by carbachol $7 \cdot 10^{-6}$ M, each had been added 10–15 min previously. Sotalol $5 \cdot 10^{-6}$ M present in all experiments. A. 1. Norpropafenone $3 \cdot 10^{-6}$ and $3 \cdot 10^{-6}$ M (nc). A. 2. Norpropafenone $0 \cdot 1$ M (nc), after 30 min pretreatment with dibucaine $8 \cdot 10^{-6}$ M (diba). A. 3. 1(3,4-dihydroxyphenyl)-isopropyl $3 \cdot 10^{-6}$ M (dihep). A. 4. Phenyloptine $3 \cdot 10^{-6}$ M (phe). (B) Responses of denervated strip of ca. pig ileum. Contraction of the preparation was maintained by histamine $7 \cdot 10^{-6}$ M, which had added 10 min previously. B. 1. Isoproterenol $4 \cdot 10^{-6}$ M (iso). B. 2. Isoproterenol $4 \cdot 10^{-6}$ M (iso) in presence of sotalol $5 \cdot 10^{-6}$ M (so). B. 3. Isoproterenol $4 \cdot 10^{-6}$ M (iso) in the presence of propranolol 10^{-6} M (prop).

appearance observed in the guinea pig was not seen on denervation of the rabbit jejunum. Lead, plexus-free parts were often surrounded by innervated areas, or innervated patches or sometimes left inside areas of denervated muscle (Fig. 6 B). This necessitated extra care in selection of denervated pieces for the experiments, no difficulty was encountered, however since the innervated and denervated areas could be differentiated without staining. The embedded, denervated pieces, no nervous elements at all were observed.

Contractile responses of innervated and denervated preparations. Innervated longitudinal strips from rabbit jejunum showed spontaneous contractions which were regular or sometimes periodic. These contractions were unaffected or only slightly inhibited by atropine

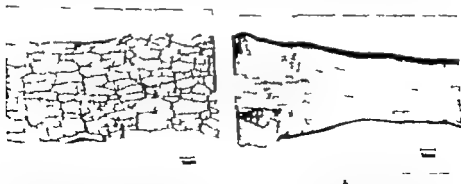


Fig. 6 (A) Photomicrograph of an innervated strip of rabbit jejunum stained with methylene blue. Numerous ganglia with primary and secondary plexuses are seen dark-stained against the paler longitudinal smooth muscle. The horizontal bar indicates 1 mm. (B) Denervated preparation from rabbit jejunum stained with methylene blue. The left patches of innervated areas are seen. The rest of the preparation is free from subserosal nerves and ganglia.

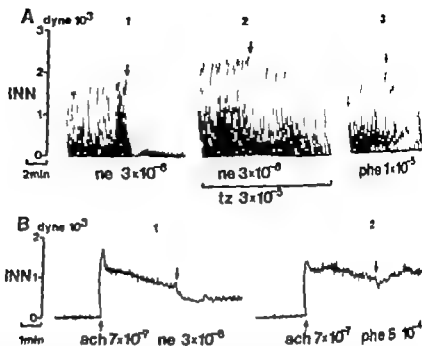


Fig. 4 (A) Responses of innervated strips of guinea pig ileum. The preparations were stimulated at 0.1 Hz. All experiments were performed in the presence of sotalol 5×10^{-8} M. A 1. Norepinephrine 3×10^{-6} M (ne). 2. Norepinephrine 3×10^{-6} M (ne) in the presence of tolazoline 3×10^{-5} M (tz). 3. Phenylephrine 1×10^{-5} M (phe). (B) Responses of an innervated strip of guinea pig ileum pretreated with sotalol 5×10^{-8} M. B 1. Acetylcholine 7×10^{-7} M (ach) and norepinephrine 3×10^{-6} M (ne). 2. Acetylcholine 7×10^{-7} M (ach) and phenylephrine 5×10^{-4} M (phe).

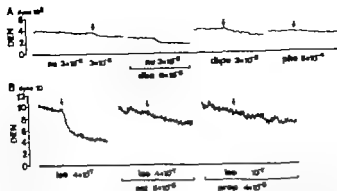
no effect on the β -adrenoceptor blocked denervated preparation. In a higher concentration (3×10^{-6} M) norepinephrine induced moderate relaxation (Fig. 5 A, 1). This relaxation however was not inhibited by any of the α -adrenoceptor antagonists tested—dibenzylamine ($4-8 \times 10^{-4}$ M) (Fig. 5 A, 2), tolazoline (1×10^{-4} M) and phentolamine (3×10^{-4} M). The effect was not influenced by propranolol (4×10^{-6} M) and neither was it inhibited if the preparation had been incubated with indomethacin (1×10^{-4} M) for one hour. The effect of norepinephrine (bi-distilled water) did not induce the effect but the norepinephrine nucleus 1-(3,4-dihydroxyphenyl)-ethanol (3×10^{-6} M) had a similar relaxant effect (Fig. 5 A, 3). The relaxation induced by norepinephrine in this high concentration was therefore probably not due to the stimulation of adrenergic receptors.

Phenylephrine in concentrations up to 5×10^{-6} M did not relax the denervated preparations (Fig. 5 A, 4).

The denervated strips were markedly relaxed by isoproterenol (4×10^{-6} M). This effect was inhibited both by sotalol (5×10^{-8} M) and by propranolol (4×10^{-6} M) (Fig. 5 A, 1-3).

Longitudinal strips from rabbit jejunum

Histological examination of methylene blue stained preparations. The innervated preparations showed a nervous plexus with an organization similar to that in guinea pig ileum (Fig. 5 B). The nervous network was somewhat more sparse than in the guinea pig and fewer ganglia and nerve bundles were identified in the embedded preparations. The typical wedge-shaped



A) Responses of denervated strips of guinea pig ileum. Contraction of the preparations was maintained by carbachol 7×10^{-6} M, which had been added 10–15 min previously. Scotalol 5×10^{-6} M was in all experiments. A, 1 Norepinephrine 3×10^{-6} and 5×10^{-6} M (ne). A, 2 Norepinephrine 5×10^{-6} M (ne), after 30 min pretreatment with dibucaine 8×10^{-4} M (dba). A, 3 1(3,4-dihydroxyphenyl)-3- 10^{-6} M (dopa). A, 4 Phenylephrine 5×10^{-6} M (phe). (B) Responses of denervated strip of pig ileum. Contraction of the preparation was maintained by histamine 7×10^{-6} M, which had been added 10 min previously. B, 1 Isoproterenol 4×10^{-6} M (iso). B, 2 Isoproterenol 4×10^{-6} M (iso) in the presence of propranolol 5×10^{-6} M (prop). B, 3 Isoproterenol 4×10^{-6} M (iso) in the presence of propranolol 10^{-6} M (prop).

variance observed in the guinea pig was not seen on denervation of the rabbit jejunum. d, plexus-free parts were often surrounded by innervated areas, or innervated patches sometimes left inside areas of denervated muscle (Fig. 6 B). This necessitated extra selection of denervated pieces for the experiments; no difficulty was encountered, however, since the innervated and denervated areas could be differentiated without staining. embedded, denervated pieces, no nervous elements at all were observed.

muscle responses of innervated and denervated preparations. Innervated longitudinal strips from rabbit jejunum showed spontaneous contractions which were regular or somewhat periodic. These contractions were unaffected or only slightly inhibited by atropine



2 (A) Photomicrograph of an innervated strip of rabbit jejunum stained with methylene blue. Numerous primary and secondary plexuses are seen dark-stained against the paler longitudinal smooth muscle. The horizontal bar indicates 1 mm. (B) Denervated preparation from rabbit jejunum stained with methylene blue. The left, patches of innervated areas are seen. The rest of the preparation is free of visible nerves and ganglia.

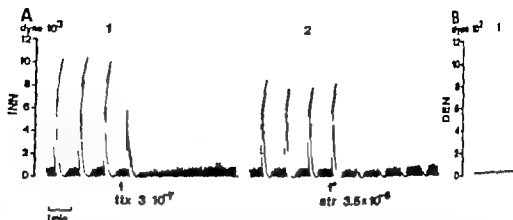


Fig. 7 (A) Response of an innervated strip of rabbit jejunum. At the dots, electrical field stimulation 10 Hz for 10 s. The arrows indicate addition of tetrodotoxin 3×10^{-7} M (tox) and atropine 3.5×10^{-6} M (atr), respectively (B) Response of a denervated strip of rabbit jejunum. Same preparation. See also calibration as in experiment 11 B, 1-2). The dots indicate electrical field stimulation at 10 Hz for 10 s.

(3.5×10^{-6} – 3.5×10^{-5} M) or by tetrodotoxin (3×10^{-7} – 3×10^{-4} M) (Fig. 7 A, 1-2). Electrical field stimulation at 1 to 10 Hz for 10 s with 1 ms pulses produced marked contractions which were usually followed by a period of inhibition. Atropine (3.5×10^{-6} – 3.5×10^{-5} M) inhibited the contractile but not the inhibitory response whereas tetrodotoxin (3×10^{-7} – 3×10^{-4} M) inhibited both responses (Fig. 7 A, 1-2). The innervated preparation was contracted by acetylcholine (7×10^{-7} M), histamine (7×10^{-6} M) and eserine (5×10^{-6} M) (Fig. 8 A, 1-3). Denervated rabbit preparations showed no or very little spontaneous activity when it occurred it was weak and irregular. The preparations failed to contract on tetanic field stimu-

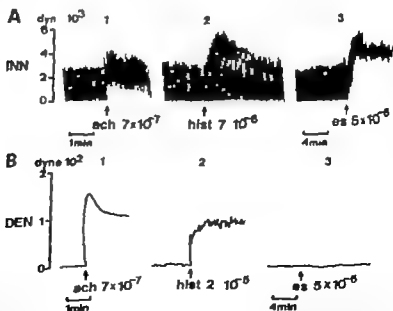


Fig. 8 (A) Responses of an innervated strip of rabbit jejunum. A 1-3: Acetylcholine 7×10^{-7} M (ach), histamine 7×10^{-6} M (hist) and eserine 5×10^{-6} M (es), respectively (B) Responses of denervated strip of rabbit jejunum. B 1-3: Acetylcholine 7×10^{-7} M (ach), histamine 2×10^{-6} M (hist) and eserine 5×10^{-6} M (es), respectively.

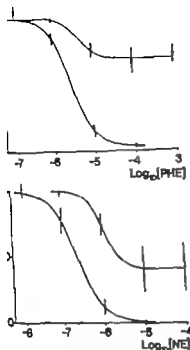


Fig. 9

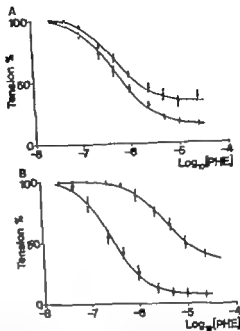


Fig. 10

9 (A) Dose-response curves of the effect of phentylephrine (PHE) in the presence of propranolol 4×10^{-6} M on the innervated rabbit jejunum. Phentylephrine was added 30 s before stimulation with either tylosine or its electric current. ● Contraction by acetylcholine 7×10^{-6} M. ○ Electrical field stimulation at 1 Hz for 10 s each run. The continuous sigmoid curves represents the regression line of the logistic dose to the experimental data. Each point represents the mean \pm S.E. of five experiments. (B) A similar experiment to 9 A but norepinephrine (NE) as used instead of phentylephrine. ● Contraction effected by tylosine 7×10^{-6} M. ○ Electrical field stimulation at 1 Hz for 10 s each run ($n=6$).

10 (A) Dose-response curves of the inhibitory effect of phentylephrine (PHE) on innervated strips of the jejunum. Contraction of the preparations maintained by eserine 1×10^{-4} M. ● Contraction maintained by acetylcholine 1×10^{-6} M ($n=6$). (B) A similar experiment to 10 A. ○ Contraction of the preparations maintained by eserine 6×10^{-6} M. ● Contraction by acetylcholine 1×10^{-6} M in the presence of propranolol 2×10^{-6} M ($n=6$).

20 (Fig. 7 B, 1 and 12, 3). Denervated preparations were contracted by acetylcholine (1×10^{-6} M) and histamine (7×10^{-6} – 2×10^{-6} M) but responded only very slightly to eserine (1×10^{-4} M) (Fig. 8 B, 1, 3 and 12, 1, 2). Before any experiments were performed on the denervated preparations they were always tested with acetylcholine (7×10^{-6} M) and eserine (5×10^{-4} M). The contraction induced by eserine on denervated strips was $4.2 \pm 0.9\%$ ($n=12$) of that induced by acetylcholine. The corresponding value in the innervated strips was $113 \pm 3\%$ ($n=5$).

Alpha-adrenoceptor mediated relaxation in innervated strips. Dose-response curves of the inhibitory effects of norepinephrine and phentylephrine were obtained from innervated rabbit jejunal strips both during contraction with electrical field stimulation for 10 s at 1 Hz and after treatment with acetylcholine (7×10^{-6} M) (Fig. 9 A, B). Propranolol (4×10^{-6} M)

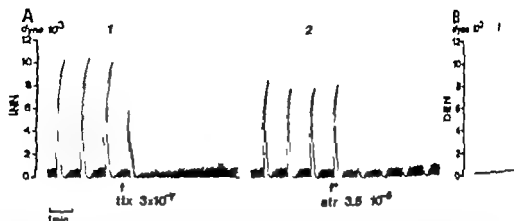


Fig. 7 (A) Response of an innervated strip of rabbit jejunum. At the dots, electrical field stimuli, 10 Hz for 10 s. The arrows indicate addition of tetrodotoxin 3×10^{-7} M (ttx) and atropine 3.5×10^{-6} M (atr), respectively. (B) Response of a denervated strip of rabbit jejunum. Same preparation and equal calibration as in experiment 11 B, 1. The dots indicate electrical field stimulation at 10 Hz for 10 s.

(3.5×10^{-6} – 3.5×10^{-5} M) or by tetrodotoxin (3×10^{-7} – 3×10^{-4} M) (Fig. 7 A, 1–2). Electrical field stimulation at 1 to 10 Hz for 10 s with 1 ms pulses produced marked contractions which were usually followed by a period of inhibition. Atropine (3.5×10^{-6} – 3.5×10^{-5} M) inhibited the contractile but not the inhibitory response whereas tetrodotoxin (3×10^{-7} – 3×10^{-4} M) inhibited both responses (Fig. 7 A, 1–2). The innervated preparation was contracted by acetylcholine (7×10^{-7} M), histamine (7×10^{-6} M) and eserine (5×10^{-6} M) (Fig. 8 A, 1–3).

Denervated rabbit preparations showed no or very little spontaneous activity. When it occurred it was weak and irregular. The preparations failed to contract on tetanic field stimu-

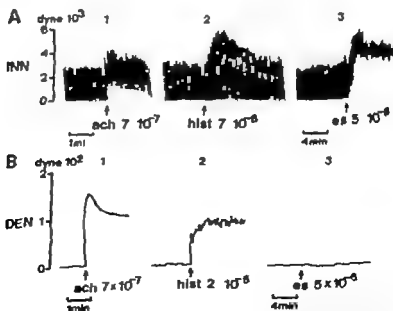
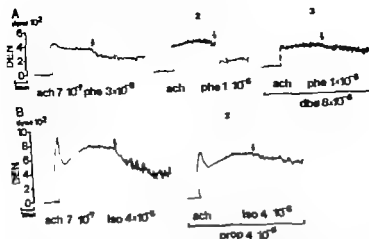


Fig. 8 (A) Responses of innervated strip of rabbit jejunum. A 1–3 Acetylcholine 7×10^{-7} M (ach), histamine 7×10^{-6} M (hist) and eserine 5×10^{-6} M (es), respectively. (B) Responses of denervated strip of rabbit jejunum. B 1–3 Acetylcholine 7×10^{-7} M (ach), histamine 2×10^{-6} M and eserine 5×10^{-6} M (es), respectively.



1) Responses of denervated strip of rabbit jejunum. A 1-2: Acetylcholine $7 \cdot 10^{-7}$ M (ach) and phenylephrine $3 \cdot 10^{-6}$ M (phe) in the presence of sotalol $5 \cdot 10^{-4}$ M (A); 3: Acetylcholine (ach) and phenylephrine $1 \cdot 10^{-6}$ M (phe) in the presence of dibenamine $8 \cdot 10^{-4}$ M (dib) and 10^{-4} M (B) Responses of denervated strip of rabbit jejunum B 1: Acetylcholine $7 \cdot 10^{-7}$ M and isoproterenol $4 \cdot 10^{-6}$ M (iso) B 2: Acetylcholine $7 \cdot 10^{-7}$ M (ach) and isoproterenol $4 \cdot 10^{-6}$ M in presence of propranolol $4 \cdot 10^{-6}$ M (prop).

(10^{-4} M) relaxed the denervated preparation contracted by angiotensin (1-2), carbachol ($7 \cdot 10^{-7}$ M) (Fig. 12, 4-5) or acetylcholine ($7 \cdot 10^{-7}$ M). If the preparation contracted by angiotensin the relaxation induced by norepinephrine was complete basal tone level (Fig. 12, 4). The relaxation induced by norepinephrine was blocked by tolazoline ($5 \cdot 10^{-6}$ M) (Fig. 12, 6) or by dibenamine ($8 \cdot 10^{-4}$ M). After blocking α -receptors with sotalol ($5 \cdot 10^{-4}$ M) oxymetazoline ($2 \cdot 10^{-6}$ M) relaxed the denervated zone contracted by carbachol ($7 \cdot 10^{-7}$ M). The relaxation induced by oxymetazoline blocked by dibenamine ($8 \cdot 10^{-4}$ M).

rotarolol ($4 \cdot 10^{-6}$ M) relaxed the denervated preparation contracted by acetylcholine relaxing effect was blocked by sotalol ($5 \cdot 10^{-4}$ M) and by propranolol ($4 \cdot 10^{-6}$ M) (Fig. 12, 1-3).

Discussion

Preparation of guinea pig ileum

Preparation of longitudinal muscle strips from the guinea pig and rabbit intestine depends on anatomical features of these organs.

The fine structure of the guinea pig ileum has been thoroughly mapped out by electron microscopic investigations (Gabella 1977 a, b). In the ileum of this animal the region between the longitudinal and circular muscle layers is occupied by the myenteric plexus, consisting of ganglia and connecting strands of nerve fibres, together with interstitial cells and blood vessels. In the circular muscle, there are numerous nerve fibres, which mainly run parallel to the muscle fibres. The nerves contain myelinated structures with several types of myelinated axons similar to those found in myelinated nerve processes within the ganglia. At some

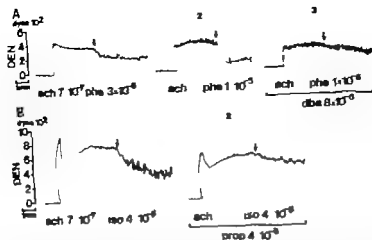
was present throughout the experiments in order to block β -adrenoceptors. The drug was added 30 s before the preparations was stimulated. The maximal inhibitory actions of norepinephrine ($100.1 \pm 0.3\%$) and phenylephrine ($95.5 \pm 1.7\%$) were significantly greater if the preparation was contracted by electrical stimulation than when it was contracted by acetylcholine ($61.9 \pm 14.0\%$ $p < 0.05$ and $37.3 \pm 4.7\%$ $p < 0.001$ respectively). The ED_{50} of the inhibitory action of norepinephrine during electrical stimulation was $3.1 \pm 0.3 \times 10^{-6}$ M but after contraction with acetylcholine it was significantly greater ($2.0 \pm 0.3 \times 10^{-6}$ M $p < 0.02$). The corresponding ED_{50} values for phenylephrine were $2.8 \pm 0.3 \times 10^{-6}$ M and 1.7×10^{-6} M respectively ($p < 0.1$).

The differences in the potencies and efficacies of norepinephrine and phenylephrine between their inhibition of contractions induced by electrical stimulation and by exogenous acetylcholine might indicate that the drugs were able to inhibit the release of acetylcholine from cholinergic neurons in the preparations. To further investigate this question a comparison was set up to investigate how phenylephrine was able to relax the innervated preparation contracted by eserine (1×10^{-6} M) and by acetylcholine (1×10^{-6} M) (Fig. 10 A). The maximal relaxation induced by phenylephrine was significantly greater after eserine contraction ($88.1 \pm 1.9\%$) than after contraction by acetylcholine ($67.2 \pm 4.4\%$ $p < 0.01$). The difference was not large and the ED_{50} of eserine and acetylcholine were similar ($5.4 \pm 0.7 \times 10^{-6}$ M and $5.6 \pm 0.7 \times 10^{-6}$ M respectively). Since the added acetylcholine might have stimulated neurons mediating a contractile action, these experiments were repeated after preparations contracted by acetylcholine had been pretreated by the ganglionic blocking agent pentolinium (2×10^{-6} M). The eserine concentration was lowered to 6×10^{-7} M in order to obtain a similar degree of contraction in the two series (Fig. 10 B). Under these conditions the ED_{50} of phenylephrine after contraction by eserine ($3.3 \pm 0.5 \times 10^{-6}$ M) was significantly lower than after treatment with acetylcholine in the presence of pentolinium (3.6×10^{-6} M $p < 0.001$). The maximal inhibition caused by phenylephrine was also significantly higher after treatment with eserine ($92.8 \pm 2.9\%$) than after the combination of acetylcholine and pentolinium ($70.3 \pm 4.8\%$ $p < 0.02$).

During contraction by acetylcholine (1×10^{-6} M) the relaxing action of phenylephrine at a concentration of up to 1×10^{-5} M was completely inhibited by dibenamine (8×10^{-6} M). At a still higher concentration (2.5×10^{-5} M) phenylephrine had a weak relaxing action which was blocked by the combination of dibenamine (8×10^{-6} M) and sotalol (5×10^{-6} M). The inhibitory effect of phenylephrine (1×10^{-5} M) during electrical stimulation was blocked by dibenamine (8×10^{-6} M).

Relaxant responses in denervated preparations. When the denervated preparation was contracted by acetylcholine (7×10^{-6} M) it was relaxed by phenylephrine (3×10^{-6} to 1×10^{-5} M) (Fig. 11 A, 1-2). The relaxation was blocked by dibenamine ($4-8 \times 10^{-6}$ M) but not by sotalol (5×10^{-6} M) (Fig. 11 A, 3-4). Phenylephrine also had a relaxing action in preparations contracted by carbachol (7×10^{-6} M), histamine (7×10^{-6} M) and angiotensin (1×10^{-6} M). The relaxing action of phenylephrine was not affected by morphine ($1-9 \times 10^{-6}$ M) or doxan (3×10^{-6} M). After contraction of the preparation by histamine or angiotensin the relaxing effect of phenylephrine was not changed by atropine (3.5×10^{-6} M).

In the presence of sotalol (5×10^{-6} M) or propranolol (4×10^{-6} to 1×10^{-5} M) no



(A) Responses of denervated strip of rabbit jejunum. A. 1 2 Acetylcholine $7 \cdot 10^{-7}$ M (ach) and phentolamine $3 \cdot 10^{-6}$ M (phe) in the presence of sotalol $5 \cdot 10^{-6}$ M. A. 3 Acetylcholine $7 \cdot 10^{-7}$ M (ach) and phentolamine $1 \cdot 10^{-5}$ M (phe) in the presence of dibenamine $5 \cdot 10^{-6}$ M (dba) and $5 \cdot 10^{-6}$ M. (B) Responses of denervated strip of rabbit jejunum. B. 1 Acetylcholine $7 \cdot 10^{-7}$ M and isoproterenol $4 \cdot 10^{-6}$ M (iso) B. 2 Acetylcholine $7 \cdot 10^{-7}$ M (ach) and isoproterenol $4 \cdot 10^{-6}$ M in the presence of propranolol $4 \cdot 10^{-6}$ M (prop)

ne ($2 \cdot 10^{-6}$ M) relaxed the denervated preparation contracted by angiotensin ($1-2$ M), carbachol ($7 \cdot 10^{-7}$ M) (Fig. 12, 4-5) or acetylcholine ($7 \cdot 10^{-7}$ M). If the preparation contracted by angiotensin the relaxation induced by norepinephrine was complete to basal tone level (Fig. 12, 4). The relaxation induced by norepinephrine was blocked by tolazoline ($5 \cdot 10^{-6}$ M) (Fig. 12, 6) or by dibenamine ($8 \cdot 10^{-6}$ M). After blocking receptors with sotalol ($5 \cdot 10^{-6}$ M) oxymetazoline ($2 \cdot 10^{-6}$ M) relaxed the denervated preparation contracted by carbachol ($7 \cdot 10^{-7}$ M). The relaxation induced by oxymetazoline blocked by dibenamine ($8 \cdot 10^{-6}$ M).

Isoproterenol ($4 \cdot 10^{-6}$ M) relaxed the denervated preparation contracted by acetylcholine. This relaxing effect was blocked by sotalol ($5 \cdot 10^{-6}$ M) and by propranolol ($4 \cdot 10^{-6}$ M) (Fig. 11 B, 1 2).

Discussion

Isolation of guinea pig ileum

Isolation of longitudinal muscle strips from the guinea pig and rabbit intestine depends on the anatomical features of these organs.

The fine structure of the guinea pig ileum has been thoroughly mapped out by electron microscopic investigations (Gabella 1972 a, b). In the ileum of this animal the region between the longitudinal and circular muscle layers is occupied by the myenteric plexus, consisting of ganglia and connecting strands of nerve fibres, together with interstitial cells and blood vessels. In the circular muscle, there are numerous nerve fibres, which mainly run parallel to the muscle fibres. The nerves contain arborose structures with several types of vesicles similar to those found in varicosities of nerve processes within the ganglia. At some

was present throughout the experiments in order to block β -adrenoceptors. The drug was added 30 s before the preparations was stimulated. The maximal inhibitory actions of norepinephrine ($100.1 \pm 0.3\%$) and phenylephrine ($95.5 \pm 1.7\%$) were significantly greater if the preparation was contracted by electrical stimulation than when it was contracted by acetylcholine ($61.9 \pm 14.0\%$, $p < 0.05$ and $37.3 \pm 4.7\%$, $p < 0.001$ respectively). The ED_{50} of the inhibitory action of norepinephrine during electrical stimulation was $3.1 \cdot 10^{-6}$ M but after contraction with acetylcholine it was significantly greater ($2.0 \pm 0.3 \cdot 10^{-6}$ M, $p < 0.02$). The corresponding ED_{50} values for phenylephrine were $2.8 \pm 0.3 \cdot 10^{-6}$ M and $1.7 \cdot 10^{-6}$ M respectively ($p < 0.1$).

The differences in the potencies and efficacies of norepinephrine and phenylephrine between their inhibition of contractions induced by electrical stimulation and by exogenous acetylcholine might indicate that the drugs were able to inhibit the release of acetylcholine from cholinergic neurons in the preparations. To further investigate this question a comparison was set up to investigate how phenylephrine was able to relax the innervated preparation contracted by eserine ($1 \cdot 10^{-6}$ M) and by acetylcholine ($1 \cdot 10^{-4}$ M) (Fig. 10 A). The maximal relaxation induced by phenylephrine was significantly greater after eserine contraction ($88.1 \pm 1.9\%$) than after contraction by acetylcholine ($67.2 \pm 4.4\%$, $p < 0.01$). The difference was not large and the ED_{50} of eserine and acetylcholine were similar ($5.4 \pm 0.7 \cdot 10^{-6}$ M and $5.6 \pm 0.7 \cdot 10^{-6}$ M respectively). Since the added acetylcholine might have stimulated neurons mediating a contractile action, these experiments were repeated after preparations contracted by acetylcholine had been pretreated by the ganglionic blocking agent pentolinium ($2 \cdot 10^{-6}$ M). The eserine concentration was lowered to $6 \cdot 10^{-6}$ M in order to obtain a similar degree of contraction in the two series (Fig. 10 B). Under these conditions the ED_{50} of phenylephrine after contraction by eserine ($3.3 \pm 0.5 \cdot 10^{-6}$ M) was significantly lower than after treatment with acetylcholine in the presence of pentolinium ($1.6 \cdot 10^{-6}$ M, $p < 0.001$). The maximal inhibition caused by phenylephrine was also significantly higher after treatment with eserine ($92.8 \pm 2.9\%$) than after the combination of acetylcholine and pentolinium ($70.3 \pm 4.8\%$, $p < 0.02$).

During contraction by acetylcholine ($1 \cdot 10^{-4}$ M) the relaxing action of phenylephrine at a concentration of up to $1 \cdot 10^{-5}$ M was completely inhibited by dibenamine ($8 \cdot 10^{-6}$ M). At a still higher concentration ($2.5 \cdot 10^{-5}$ M) phenylephrine had a weak relaxing effect which was blocked by the combination of dibenamine ($8 \cdot 10^{-6}$ M) and sotalol ($5 \cdot 10^{-6}$ M). The inhibitory effect of phenylephrine ($1 \cdot 10^{-5}$ M) during electrical stimulation was blocked by dibenamine ($8 \cdot 10^{-6}$ M).

Relaxant responses in denervated preparations When the denervated preparation was contracted by acetylcholine ($7 \cdot 10^{-5}$ M) it was relaxed by phenylephrine ($3 \cdot 10^{-5}$ – $1 \cdot 10^{-4}$ M) (Fig. 11 A, 1–2). The relaxation was blocked by dibenamine (4 – $8 \cdot 10^{-6}$ M) but not by sotalol ($5 \cdot 10^{-6}$ M) (Fig. 11 A, 2–3). Phenylephrine also had a relaxing action in preparations contracted by carbachol ($7 \cdot 10^{-5}$ M), histamine ($7 \cdot 10^{-6}$ M) and angiotensin ($1 \cdot 10^{-6}$ M). The relaxing action of phenylephrine was not affected by morphine ($1.9 \cdot 10^{-5}$ M) or clonidine ($3 \cdot 10^{-6}$ M). After contraction of the preparation by histamine or angiotensin the relaxing effect of phenylephrine was not changed by atropine ($3.5 \cdot 10^{-6}$ M).

In the presence of sotalol ($5 \cdot 10^{-6}$ M) or propranolol ($4 \cdot 10^{-5}$ – $1 \cdot 10^{-4}$ M) non-

copy using silver impregnation. Between the longitudinal and circular muscle are angia of Auerbach's plexus. Nerve fibres running in this region consist of the largest myelinated bundles dividing up into a finer secondary and tertiary plexus. From the tertiary plexus very fine nerve bundles together with blood capillaries and interstitial cells enter the muscle and ramify between smooth muscle cells of both longitudinal and circular muscle. Richardson observed that when the circular muscle was stripped from the longitudinal muscle during the preparation of the tissue, the tertiary plexus was often damaged or completely removed from the longitudinal muscle.

The methylene blue stained innervated rabbit jejunum used in this study ganglia and primary and secondary nerve bundles were observed, as in guinea pig ileum. The denervated strips lacked these nervous elements (Fig. 6 A, B). As with guinea pig ileum it could not be concluded that small nerves not visible in the stereo microscope remained in the preparation.

The innervated preparations showed a biphasic response on electrical stimulation. First a small contraction followed by an inhibition of the spontaneous motility. Since the contractions were abolished by atropine they were probably mediated by acetylcholine released from the cholinergic neurons of the preparations. The inhibitory response to electrical stimulation was not affected by atropine but was probably of nervous origin, since it was abolished by tetrodotoxin. The denervated preparations did not respond to electrical field stimulation but were contracted by agents such as acetylcholine, carbachol and histamine which are considered to act directly on smooth muscle. In the denervated preparations eserine induced only a minute response in comparison with that resulting from acetylcholine. The innervated preparations on the other hand, responded by a similar degree of contraction to both drugs.

Since the rabbit small intestine seems to be deeply innervated by small nerve fibres, clearly demonstrable in the electron microscope (Richardson 1958), it is probable that the denervation procedure used in this study leaves the intramuscular nerve endings intact while the ganglia and larger nerves—and probably the tertiary nerve plexus located between the longitudinal and circular muscle—are removed. The small response to eserine observed in the denervated preparations is probably due to a potentiation of the action of acetylcholine released from the intramuscular nerve endings left in the preparations. These nerves, however, are not able to induce contractions of the muscle on electrical stimulation at the same strength, frequency and duration of impulses that contracted the innervated preparations. The functional ability of the nerve endings left in the preparations was thus so impaired that they were unable to contract the preparation to any appreciable extent. Under such conditions a relaxing mechanism acting on the neurons could only affect the tone of the smooth muscle to a very limited degree.

Adrenergic receptors in longitudinal guinea pig ileum

The innervated strips from guinea pig ileum possessed α -receptors capable of inducing an inhibitory response. Norepinephrine in low concentrations inhibited electrical field stimulation at 0.1 Hz. The contractions following electrical stimulation at this frequency were probably mediated by the activation of cholinergic neurons, since they were abolished by atro-

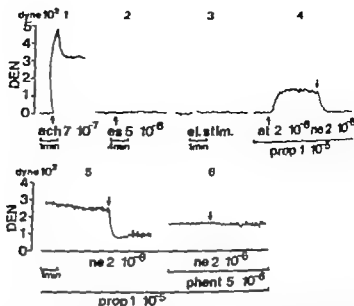


Fig. 1. Responses of a denervated strip from rabbit jejunum. 1 Acetylcholine $7 \cdot 10^{-7}$ M (ach). 2 Eserine $5 \cdot 10^{-6}$ M (es). 3 Electrical field stimulation by 1 ms rectangular pulses during 10 s at 25 Hz (O) and at 50 Hz (*). 4 Angiotensin $2 \cdot 10^{-6}$ M (at) and norepinephrine $2 \cdot 10^{-6}$ M (ne) in presence of propranolol $1 \cdot 10^{-6}$ M (prop). 5 Tension of the preparation increased by carbachol ($1 \cdot 10^{-6}$ M) added 10 min previously. At arrow norepinephrine $2 \cdot 10^{-6}$ M (ne) in the presence of propranolol $1 \cdot 10^{-6}$ M. 6. Tension of the preparation increased by carbachol added 10 min previously. At arrow norepinephrine $2 \cdot 10^{-6}$ M (ne) in the presence of propranolol $1 \cdot 10^{-6}$ M and phentolamine $5 \cdot 10^{-6}$ M (phen).

points ganglia approach the surface of the longitudinal muscle and at other points fibres and interstitial cells are interposed between the ganglia and the smooth muscle but these structures never seem to penetrate into this muscle layer.

In the innervated preparations used in this study ganglia and primary and secondary nerve bundles were observed in the stereo microscope after methylene blue staining. Denervated pieces clearly lacked these structures (Fig. 1 A, B). The possibility cannot be excluded that smaller nervous structures beyond the resolving power of the microscope remained in the preparations. Evidence of complete denervation or at least complete functional denervation of the preparations must therefore be supported by other criteria.

The denervated preparations did not respond to eserine, nicotine or electrical field stimulation while they were still contracted by drugs such as acetylcholine, carbachol and histamine. The innervated preparations, on the other hand, responded to all these stimuli. These findings are in accordance with the results of Paton (1968) who also demonstrated that denervation of guinea pig ileum lowered the acetylcholine content to less than 1% of innervated controls. The interpretation of these findings together with the knowledge of the structural organization of autonomic nerves in the longitudinal guinea pig ileum clearly indicate that denervation yielded a preparation in which the nervous function was lost.

Innervation of rabbit jejunum

In rabbit intestine the organization of the autonomic nerves is basically similar to that in guinea pig. Richardson (1958, 1960) described the microanatomy of the rabbit small intestine as elucidated by electron microscopy with a potassium permanganate technique and

copy using silver impregnation. Between the longitudinal and circular muscle are plexuses of Auerbach's plexus. Nerve fibres running in this region consist of the largest nerve bundles dividing up into a finer secondary and tertiary plexus. From the tertiary very fine nerve bundles together with blood capillaries and interstitial cells enter a tissue and ramify between smooth muscle cells of both longitudinal and circular muscle. Richardson observed that when the circular muscle was stripped from the longitudinal muscle during the preparation of the tissue the tertiary plexus was often damaged or completely removed from the longitudinal muscle.

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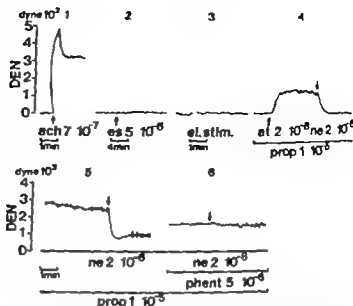


Fig. 12. Responses of a denervated strip from rabbit jejunum. 1 2 Acetylcholine $7 \cdot 10^{-7}$ M (sch). 3 Eserine $5 \cdot 10^{-6}$ M (es). 4 Angiotensin $2 \cdot 10^{-8}$ M (at) and norepinephrine $2 \cdot 10^{-6}$ M (ne) in the presence of propranolol $1 \cdot 10^{-5}$ M (prop). 5 Tension of the preparation increased by carbachol $1 \cdot 10^{-4}$ M added 10 min previously. At arrow norepinephrine $2 \cdot 10^{-6}$ M (ne) in the presence of prop $1 \cdot 10^{-5}$ M. 6. Tension of the preparation increased by carbachol added 10 min previously. Norepinephrine $2 \cdot 10^{-6}$ M (ne) in the presence of propranolol $1 \cdot 10^{-5}$ M and phenolamine $5 \cdot 10^{-6}$ M (phen).

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Adrenergic receptors in longitudinal guinea pig ileum

The innervated strips from guinea pig ileum possessed α -receptors capable of inducing an inhibitory response. Norepinephrine in low concentrations inhibited electrical field stimulation at 0.1 Hz. The contractions following electrical stimulation at this frequency were probably mediated by the activation of cholinergic neurons, since they were abolished by atropine.

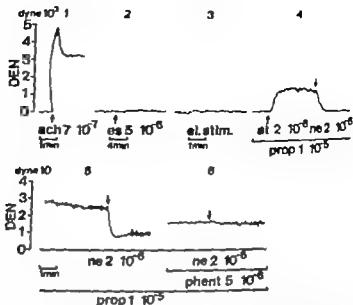


Fig. 12. Responses of a denervated strip from rabbit jejunum. 1. Acetylcholine 7×10^{-4} M (es). 2. Esclatine 5×10^{-6} M (es). 3. Electrical field stimulation by 1 ms rectangular pulses during 10 s at 25 Hz (O) and at 50 Hz (*). 4. Angiotensin 2×10^{-6} M (i) and norepinephrine 2×10^{-4} M (ne) in presence of propranolol 1×10^{-5} M (prop). 5. Tension of the preparation increased by carbachol 1×10^{-4} M added 10 min previously. At arrow norepinephrine 2×10^{-4} M (ne) in the presence of prop 1×10^{-5} M. 6. Tension of the preparation increased by carbachol added 10 min previously. Norepinephrine 2×10^{-4} M (ne) in the presence of propranolol 1×10^{-5} M and phentolamine 5×10^{-6} M (phent).

points ganglia approach the surface of the longitudinal muscle and at other points fibres and interstitial cells are interposed between the ganglia and the smooth muscle but these structures never seem to penetrate into this muscle layer.

In the innervated preparations used in this study ganglia and primary and secondary nerve bundles were observed in the stereo microscope after methylene blue staining. Denervated pieces clearly lacked these structures (Fig. 1 A, B). The possibility cannot be excluded that smaller nervous structures beyond the resolving power of the microscope remained in the preparations. Evidences of complete denervation or at least complete denervation of the preparations must therefore be supported by other criteria.

The denervated preparations did not respond to eserine, nicotine or electrical field stimulation while they were still contracted by drugs such as acetylcholine, carbachol and histamine. The innervated preparations, on the other hand, responded to all these stimuli. The findings are in accordance with the results of Paton (1968) who also demonstrated that denervation of guinea pig ileum lowered the acetylcholine content to less than 1/4 of the innervated controls. The interpretation of these findings together with the knowledge of the structural organization of autonomic nerves in the longitudinal guinea pig ileum indicate that denervation yielded a preparation in which the nervous function was almost completely abolished.

Innervation of rabbit jejunum

In rabbit intestine the organization of the autonomic nerves is basically similar to that in guinea pig. Richardson (1958, 1960) described the microanatomy of the rabbit small intestine as elucidated by electron microscopy with a potassium permanganate technique and

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Innervated strip from the rabbit was relaxed by α -receptor stimulation. This relaxation had been mediated through adrenergic α -receptors located in the smooth muscle and/or through α -receptors located in the neural structures of the preparation. It was by Vin and Knoll (1971) that norepinephrine inhibited the release of acetylcholine in the rabbit jejunum under resting conditions or during electrical stimulation at low frequency (0.5 Hz). It is probable that such an inhibitory effect on cholinergic neurons could tribute to the action of the adrenergic drugs on the innervated rabbit jejunum. If this is so, the response to α -receptor stimulation would be greater on contraction of the preparation by electrical stimulation of cholinergic neurons than on contraction by exogenous acetylcholine, which mainly acts directly on smooth muscle. It was found that both norepinephrine and phenylephrine, in the presence of propranolol, were more effective in inhibiting electrical contractions at a low stimulation frequency (1 Hz) than they were in inhibiting contractions induced by exogenous acetylcholine. Similarly phenylephrine had a stronger relaxing effect on preparations contracted by eserine than on those contracted by exogenous acetylcholine. It is probable that contractions elicited by acetylcholine in the innervated preparation were to some extent mediated through the stimulation of neurons. In one experiment the ganglionic blocker pentolinium was used to inhibit this action of acetylcholine on neurons. Under these experimental conditions phenylephrine was markedly less potent as a relaxing agent when the preparation was contracted by acetylcholine in the presence of pentolinium than when it was contracted by eserine. From these experiments it can be concluded that the receptor mediated relaxation in innervated rabbit jejunum is at least partially brought about through inhibition of the intrinsic cholinergic neurons of the preparation.

The denervated strip from the rabbit contained α -receptors mediating a relaxant response. Innervated preparations contracted by several agents such as acetylcholine were relaxed by phenylephrine, norepinephrine and oxymetazoline. These responses were still observed after addition of the β -blocking agent sotalol, but they were blocked by dibenamine indicating that stimulation of adrenergic α -receptors had occurred. It is unlikely that an action of the nerve endings not removed by the denervation procedure could have been responsible for the relaxant effect of the adrenergic drugs. The denervated preparation did not contract in response to electrical stimulation and responded only very slightly to eserine. Under these conditions, the contraction induced by acetylcholine or other drugs acting directly on smooth muscle could not have been mediated through neurons to any appreciable extent. Nevertheless, the denervated preparation was markedly relaxed by α -adrenergic agonists. In the case of norepinephrine, on the angiotensin contracted preparation, this relaxation was complete, to the basal tone of the preparation. Moreover the responses to the adrenergic agonists were not affected by tetrodotoxin or by atropine when the preparations had been contracted by norepinephrine or angiotensin. Thus, it is suggested from these experiments that the in the rabbit longitudinal jejunum α -receptors are located both in the smooth muscle cells and in the cholinergic neurons of the nerve plexus of the smooth muscle.

The denervated rabbit jejunum was relaxed by isoproterenol in low concentrations. This relaxing action of isoproterenol was blocked by sotalol or propranolol, indicating an action on β -adrenoceptors located in smooth muscle cells.

pine. The response to norepinephrine was not affected by β -adrenoceptor blockade inhibited by tolazoline, indicating an involvement of α -adrenoceptors. When the innervated strips had been contracted by acetylcholine a relaxing effect was exerted by norepinephrine and sometimes by phenylephrine. It is probable that the contraction induced by acetylcholine in the innervated strips was, to some extent, mediated through the stimulation of α -adrenoceptors.

Strips of denervated longitudinal ileum from the guinea pig contracted by acetylcholine or other drugs were not relaxed by norepinephrine in the same concentration that the innervated strips. Nor were the preparations relaxed by phenylephrine in concentrations of up to 5×10^{-4} M after blockade of β -adrenoceptors. Norepinephrine in concentrations (3×10^{-5} M) relaxed the preparations, however. This effect was not seen as a stimulation of α -adrenoceptors, since it was resistant to α -adrenoceptor blocking agents. Nor was it mediated by β -adrenoceptors, since neither β -receptor blocking agents nor a combination of α and β -blockers abolished the response. Involvement of prostaglandins in the production of this effect of norepinephrine was also improbable, since inhibition of prostaglandin synthesis by indomethacin did not affect the response. Since a relaxant effect was produced by 1-(3,4-dihydroxyphenyl)-ethanol in the same molar concentration, the effect was probably not mediated by adrenoceptors but by an unspecific mechanism dependent on the nucleus of the catecholamine.

These results indicate the absence of inhibitory α -receptors in the smooth muscle of the denervated guinea pig ileum. The inhibitory response observed in innervated preparations following α -receptor stimulation must therefore be due to an action on nervous neurons, probably of a cholinergic nature.

Adrenergic α -receptors, excitatory or inhibitory from different species generally show great homogeneity with respect to the affinity constants and the activities of α -adrenergic active drugs (Furchgott 1972). A difference has recently been observed between α -adrenoceptors located in the cholinergic neurons of Auerbach's plexus of guinea pig ileum and the guinea-pig ileocaecal sphincter and the excitatory α -adrenoceptors located in the smooth muscle cells of the guinea pig ileocaecal sphincter and rabbit aorta (Wikberg & Wikberg *et al.* 1975). The presence of a pharmacologically different α -adrenoceptor in the guinea pig ileum explains a variety of hitherto conflicting data. Phenylephrine is regarded as a specific α -adrenergic agonist. This is the case when the drug is tested on the jejunum or rabbit aorta. The drug had virtually no or very little effect, however, when tested on electrically contracted guinea pig ileum or guinea pig ileocaecal sphincter (Wikberg & Wikberg *et al.* 1975; Wikberg 1977). Paton and Vizi (1969) reported that phenylephrine was comparatively inactive in reducing the release of acetylcholine following electrical stimulation in guinea pig ileum. Phenylephrine, like other 3-substituted hydroxy-phenylamines, was almost completely ineffective in inhibiting the peristaltic reflex in guinea pig ileum (McDoughal and West 1954). The weak and transient effects produced by phenylephrine on the innervated preparations of the guinea pig in this study should be considered in the light of the above discussion.

The denervated guinea pig ileum was relaxed by isoproterenol. The relaxing action of isoproterenol was blocked by sotalol or propranolol, indicating an action on the β -adrenoceptors located in the smooth muscle cells.

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and GHR) the perhaps dominating genetic predisposition to hypertension seems to be of a central neurohormonal nature, where an increased sympathetic activity and/or reaction to extrinsic stimuli, act in such a way as to cause an age-dependent, progressive blood pressure elevation.

As in SHR there is good evidence for a hyper-reactivity in the central neural structures, affecting cardiovascular responses to environmental stimuli (Halldäck and Folkow 1974 and Folkow 1975). Such, perhaps, fairly episodic but exaggerated cardiovascular responses imply an elevated mean arterial pressure, as calculated over days and weeks, which in turn causes adaptive structural changes in the high pressure sections of the cardiovascular system. In large arteries and the precapillary resistance vessels an increased wall to lumen ratio rapidly occurs which seems to be the prerequisite for a truly hypertensive state (e.g. Folkow *et al.* 1973, Folkow 1975). In fact, whichever the initiating functional disturbance, central cardiovascular adaptations, including left ventricular hypertrophy are gradually developed in all types of hypertension (*cf.* Pickering 1968). These structural vascular changes seem to reset both the range of resistance vessel control and the baroreceptor function to a new pressure equilibrium. It further implies a vascular hyper-reactivity which potentiates extant functional influences and hence creates a positive feedback interaction with these influences.

Much attention has been focussed on the possibility of a primary renal involvement, at least in some variants of essential hypertension in man or genetic hypertension in animals. However regardless of the aetiology of hypertension the kidney will sooner or later become involved, both because there are many ways in which its function can be reset and because the vascular bed seems to be particularly vulnerable, and hence disturbed, by increases in the pressure load. Although there seems to be no primary renal factor contributing to the initiation of hypertension in GHR and SHR, other strains of "spontaneously" hypertensive rats have been developed in which renal factors seem to play a primary and dominant role. Dehl, Heine and Tassinari (1962) have developed a strain of rats, "the Brookhaven strain of hypertension-sensitive rats". These rats develop hypertension when exposed to a stressor as salt load and the salt "sensitivity" can be transferred to salt-resistant rats by kidney transplantation (Dehl, Heine and Thompson 1974).

Renal factors, but evidently of somewhat different nature, also contribute to the development of hypertension in the Milan strain of hypertensive rats (MHS) (Branchi, Fox and Imbasciati 1973). Thus, MHS does not need a high salt diet to develop hypertension but compared with normotensive controls the renal filtration capacity seems to be reduced at the prehypertensive stage in relation to the tubular reabsorptive capacity. MHS exhibits signs of an increased plasma volume in early age which seems related to a modest salt retention, but when blood pressure increases these changes revert back towards normal (Branchi *et al.* 1975 a, b). Thus, it seems that the kidneys in the MHS need a higher perfusion pressure to operate in a normal way. Also, if the kidneys are cross-transplanted between MHS and their appropriate normotensive controls, the hypertensive stage goes with the kidneys from the hypertensive animals (Branchi *et al.* 1974).

The present study was undertaken to investigate some aspects of the hemodynamic situation in this MHS type of "volume hypertension". At the same time the neurogenic cardio-

Cardiovascular Control in the Milan Strain of Spontaneously Hypertensive Rat (MHS) at "Rest" and during Acute Mental "Stress"

By

MARGARETA HALLBÄCK, JOHN V JONES, GIUSEPPE BIANCHI and BRÖRN FOLKOW

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Abstract

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The cardiovascular responses to acute mental "stress" were compared in the Milan strain of spontaneously hypertensive rats (MHS) and in normotensive control rats (NR). Blood pressure and heart rate were followed in pairs of awake MHS and NR, while defence reactions were provoked by alerting stimuli (noise, vibration). No differences were noted between the two groups in response to "stress" although resting heart rate in MHS was lower than in NR. Administration of triproline or propranolol to MHS and NR showed the MHS to have higher resting vagal tone and lower sympathetic tone than the NR. Seven weeks (at least two weeks later) hemodynamic investigation, under nembutal anesthesia, showed no difference in cardiac output between MHS and NR but a higher stroke volume, presumably related to the lower heart rate in MHS. Thus, total peripheral resistance was increased in MHS as was the ratio left ventricular weight/body weight, and in good proportion to the blood pressure rise. Thus MHS differ substantially both their responses to "stress" and also hemodynamically from the Okamoto strain of spontaneously hypertensive rat (SHR), being the so far most studied and best known model of essential hypertension in man. In MHS the hypertension is more of a systolic type and is of primarily renal origin. As such, MHS provide another model for investigating the polygenic nature of hypertension in man.

The search for an adequate animal model of essential hypertension in man has led to the development of several different strains of genetically or "spontaneously" hypertensive rats. The first to be developed was the New Zealand strain of genetically hypertensive rats (GHR), based on selective breeding of Wistar rats with above normal blood pressure levels (Smirk and Hall 1958). However the most commonly used and hence most extensively studied type of genetically hypertensive rat is the spontaneously hypertensive rat (SHR) developed from the Kyoto Wistar strain by Okamoto and Aoki (1963). In both these strains

On leave from University Department of Medical Cardiology Royal Infirmary Glasgow Scotland
From Medical Clinic I, University of Milan, Italy

TABLE 1 The resting heart rate (HR) and mean arterial blood pressure (MBP) are given for both the Milan strain of spontaneously hypertensive rats (MHS) and their normotensive controls (NR). The change from resting values in one group given propranolol and another given atropine are shown. All values are mean \pm S.E.

	Resting		Change from resting value			
	MBP	HR	After propranolol		After atropine	
			MBP	HR	MBP	HR
MHS	118 \pm 2	309 \pm 6	+18 \pm 2	51 \pm 8	+1 \pm 2	+68 \pm 6
NR	107 \pm 2	351 \pm 8	+12 \pm 1	74 \pm 10	-2 \pm 1	+43 \pm 15
	-76	-16	-8	-3	-3	-8
	0.01	0.001	0.05	0.05		0.01

isolated room, and when the rats had recovered from the superficial ether anaesthesia (see Table 1). In this situation of paired comparison in identical environments a mild hypertension was evident in the MHS, the mean pressure being 118 ± 2 mmHg as compared with 107 ± 2 mmHg in NR ($p < 0.01$), i.e. 10% difference. Basal heart rate in MHS was, however, reduced by some 10 per cent as compared to NR, being 309 ± 6 beats/min and 351 ± 8 beats/min, respectively ($p < 0.001$).

When exposed to brief periods of stressful stimuli MHS and NR exhibited largely similar responses, i.e. their increases in heart rate and blood pressure did not substantially differ either in direction or extent, as shown in the lower part of Fig. 1. The mean increase in heart rate during the 30 s stimulatory period showed a tendency to be slightly more pronounced in MHS, being here 13 ± 5 beats/min compared with 6 ± 4 in NR but the difference was not statistically significant. The mean increase in blood pressure during the stress period was only 2 ± 2 mmHg in MHS and 3 ± 1 mmHg in NR. These responses are thus quite modest in extent compared with the powerful ones induced in the Okamoto strain of SHR compared with their controls, when exposed to the same stimuli (see Fig. 1 upper part).

Vagotonic control. To evaluate possible differences between MHS and NR concerning vagotonic control of the heart, the 16 pairs of rats were divided into two groups. One group (consisting of 8 MHS and 8 NR) received propranolol after having been tested in the stress box. The degree of adrenergic influence on the heart in the awake "resting" MHS and NR was judged by the individual change in heart rate after administration of propranolol. These changes were significantly smaller in MHS than in NR in this particular situation of paired comparison during rest (see Table 1). The remaining rats were given atropine. A greater increase in heart rate was then evident in MHS than in NR which thus suggested more pronounced vagal tonic influence on the MHS heart (Table 1).

Thus, the heart rate of the MHS was relatively less affected by propranolol than by atropine, whereas the reverse was true for the NR. This indicates that in the Milan strain of spontaneously hypertensive rats the heart is more influenced by vagal control than in the case in their appropriate NR, at least in the prevailing situation which was identical for both. Furthermore the sympathetic cardiac activity seems less pronounced in MHS than that present in NR. The fact that the resting heart rate in MHS in this type of paired

vascular control in MHS was explored during both basal conditions and during brief episodes of stressful stimulation. In paired comparisons with their appropriate normotensive controls.

Methods

Sixteen rats of the Milan hypertensive strain and 16 normotensive Milan Wistar rats (NR) were used in "stress" expts. For hemodynamic analysis 10 rats out of each group were used, having been earlier used for brief exposures to environmental stimuli to explore their cardiovascular reactivity. At least 4 weeks elapsed between these two types of acute expts. All rats used were males aged 4-5 months, weight between 300 and 350 g. The "stress" expts. were always carried out on pairs of one MHS and one NR. Their tail arteries were cannulated during brief ether anesthesia, the incision sutured and local anesthetic applied around the incision. The rats were placed in the stress box in which they could be exposed 30 s periods of brief stress stimulations in the form of loud high-frequency noise and/or vibrations of box (for further details see Hallbäck and Folkow 1974). The rats were allowed to recover from the effects of ether one hour before the experiment started. Blood pressure and heart rate were continuously recorded from the tail artery cannula which was connected to a Statham pressure transducer operating on a C. Polygraph. Presented mean values during basal conditions are averages of at least 3 recordings in one rat during a 40 min period prior to any stress stimulation. The changes in heart rate and blood pressure during the 30 s stress stimuli and the 30 poststimulatory s are presented as follows. The changes in prestimulatory values are calculated for each third second in each rat, after which the difference in response between the paired MHS and NR at each third second interval is calculated. The mean of these differences, and the standard error of the mean differences, are then calculated as well as the significant differences in response (see further Hallbäck and Folkow 1974). A significant difference in total response is considered to be present when 6 out of the 10 observations during stress stimuli, or 12 out of the 30 observations, differ above 95 per cent probability.

In the course of the expts. atropine, 1 mg/kg, was given to block the vagal cardiac effects and propranolol, 3 mg/kg, was given to block the sympathetic cardiac effects. The drugs were administered to the paired animals in the tail artery in no larger volume than 0.5 ml. The respective drug effects are calculated in terms of individual changes in heart rate and blood pressure in each rat from the pre-drug control value. The presented mean values and standard error of the mean, as well as level of significance, were calculated by means of paired *t*-test.

The rats used in the hemodynamic study were first briefly anesthetized with ether. Their right femoral artery and vein were then cannulated for which the rats were slowly given 50 mg/kg body weight of sodium pentobarbital as an i.v. infusion. The tail artery was cannulated for blood pressure and heart rate measurements. The cannula was connected to Statham pressure transducer writing on Grass Polygraph model 7 (kPa = 500 IU/kg b.w. was given to prevent clotting of blood. The dye dilution technique was used for cardiac output measurements. By means of a 10- μ l dispenser syringe 0.04 ml of a cardiogreen solution (2 mg/l) was rapidly injected into the femoral vein. For each recording of the arterial concentration of dye no more than 0.35 ml of blood (less than 5 per cent of the total blood volume) was sucked from the femoral artery into a specially designed low volume (0.05 ml) densitometer cuvette (for reference see Albrecht et al 1970). After each determination the blood was rapidly reinfused. The densitometer was connected to a Servo for recording of the dye dilution curves. For calculating the dye dilution recordings, a digitizer unit connected to a Hewlett Packard calculator was used. Statistical evaluation of the hemodynamic study was carried out by means of the group comparison *t*-test.

Results

Stress experiments. The cardiovascular responses to brief episodes of stressful stimulation (sudden loud noise or vibrations of the box) were tested in 16 pairs of MHS and their normotensive Milan control rats.

"Basal" values of blood pressure and heart rate prior to the stress episodes were obtained in the awake rats after 1 h accommodation to the stress-box in a semidark room.

mean pressure was 97 ± 4 mm Hg. Also during nembutal anesthesia and surgically for the cardiac output measurements, the heart rate of the MHS was significantly less than that in NR, being 320 ± 9 beats/min vs. 383 ± 9 beats/min ($p < 0.001$). There was no difference between MHS and NR concerning cardiac output, which was $3 \text{ ml/min } 100 \text{ g}$ in MHS and $21.7 \pm 1.3 \text{ ml/min } 100 \text{ g}$ in NR. Because of the lower heart rate, stroke volume was 16 per cent greater in MHS than in NR ($p < 0.05$) being $66 \pm 4 \mu\text{l}$ vs. $57 \pm 3 \mu\text{l}/100 \text{ g}$.

It follows from the mean arterial pressure and cardiac output figures that total peripheral resistance was elevated by about 15 per cent in MHS. Left ventricular weight by weight was some 15 per cent higher in MHS than in NR as a reflection of the increased afterload for the left ventricle (see Table II). It thus appears that the increase in volume, in total peripheral resistance and degree of hypertrophic adaptation of the stroke are in good proportion to the increase in mean arterial pressure in these rats with mild chronic elevation of mean arterial pressure.

Discussion

In the present experimental situation the blood pressure elevation was found to be fairly mild in MHS, as judged by only some 15 per cent increase in mean arterial blood pressure compared to control rats (NR). The technique used in the present study of direct arterial blood pressure recording from the tail artery is the standard method employed in this department. However when mean arterial pressure is instead measured *via* the carotid artery the difference between MHS and NR is in the order of some 25 per cent (Blanchi, unpublished). It thus appears as if MHS also display an increased resistance along more proximal arteries as well as along the true resistance vessels, since the difference in pressure between the carotid and tail arteries is considerably smaller in NR than in MHS.

However irrespective of such divergences in mean pressure level along the arterial tree, MHS hypertension seems to be more mild than in SHR and to involve some genetically determined renal abnormality (Blanchi, Fox and Imbasciani 1973; Blanchi *et al* 1975 a, b). The hemodynamic consequences of such renal abnormality which tends to cause an increased plasma volume, have been thoroughly discussed by e.g. Ledingham and Cohen (1964), Guyton and Coleman (1969), Blanchi, Tenconi and Lucca (1970) and Ledingham (1971). It has been suggested, that a primary overfilling of the circulatory system leads to increased cardiac filling pressure, and hence to an increased stroke volume and cardiac output. In response to the increased output "whole body auto-regulation" is assumed to take place, upon which cardiac output and blood volume return towards normal values (Granger and Coleman 1971).

The results from the present hemodynamic investigation in MHS may in general terms, follow the above-mentioned sequence of events. Thus, the increased stroke volume found in MHS, in combination with signs of a reduced sympathetic influence on the heart, suggest that a raised cardiac filling pressure was present. The increased stroke volume may explain, at least in part, why systolic pressures in MHS differ particularly much from those in NR (Blanchi *et al* 1975 a, b), while the differences in mean arterial pressure were more modest.

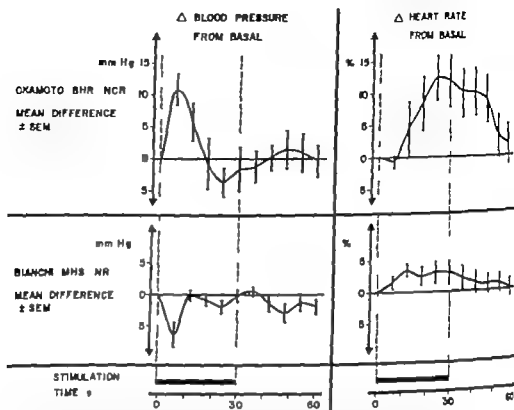


Fig. 1 Mean difference (\pm S.E.) in blood pressure (left) and heart rate (right) responses to stress stimulation between 9 pairs of SHR of the Okamoto strain and their controls, NCR (from Hallbäck 1975) and between 18 MHS rats and their controls, NR (lower panel). Changes in heart rate are expressed in per cent resting heart rate.

comparison is significantly lower than in NR may thus be due to such opposite, but evidently modest, shifts in vagal and sympathetic control of the heart in MHS.

Hemodynamic study. 10 MHS and 10 NR were investigated during nembutal anaesthesia under identical experimental circumstances. As shown in Table II mean arterial pressure was 110 ± 3 mmHg in MHS being thus elevated some 15 per cent compared to that of

TABLE II Mean \pm S.E. values for mean arterial blood pressure (MBP), heart rate (HR), cardiac output (CO), stroke volume (SV), total peripheral resistance (TPR) and left ventricular weight as a percentage of total body weight are given for both the Mann strain of spontaneously hypertensive rat (MHS) and their normotensive controls (NR). The ratios of these values in MHS/NR are given as are the statistical differences between them.

	MBP	HR	CO ml/min 100 g	SV ml/100 g	TPR mmHg/ml min 100 g	Left ventricular weight/body weight
MHS n=10	110 ± 3	320 ± 9	21.1 ± 1.3	66 ± 4	5.1 ± 0.4	0.210 ± 0.01
NR n=10	97 ± 4	303 ± 9	21.7 ± 1.3	57 ± 3	4.5 ± 0.3	0.184 ± 0.00
MHS/NR	1.13	0.84	0.97	1.16	1.15	1.14
	-p 0.05	-p 0.01	-p 0.001			

mean pressure was 97 ± 4 mm Hg. Also during nembutal anesthesia and surgically for the cardiac output measurements, the heart rate of the MHS was significantly less than in NR, being 320 ± 9 beats/min *vs.* 383 ± 9 beats/min ($p < 0.001$). There was no difference between MHS and NR concerning cardiac output, which was $13 \text{ ml/min } 100 \text{ g}$ in MHS and $21.7 \pm 1.3 \text{ ml/min } 100 \text{ g}$ in NR. Because of the lower rate, stroke volume was 16 per cent greater in MHS than in NR ($p < 0.05$) being $66 \pm 4 \text{ g}$ *vs.* $57 \pm 3 \text{ ml/100 g}$.

From the mean arterial pressure and cardiac output figures that total peripheral resistance was elevated by about 15 per cent in MHS. Left ventricular weight by weight was some 15 per cent higher in MHS than in NR as a reflection of the increased size of the left ventricle (see Table II). It thus appears that the increase in volume, in total peripheral resistance and degree of hypertrophic adaptation of the stroke are in good proportion to the increase in mean arterial pressure in these rats with mild chronic elevation of mean arterial pressure.

Discussion

In the present experimental situation the blood pressure elevation was found to be fairly mild in MHS, as judged by only some 15 per cent increase in mean arterial blood pressure compared to control rats (NR). The technique used in the present study of direct arterial blood pressure recording from the tail artery is the standard method employed in this department. However when mean arterial pressure is instead measured via the carotid artery the difference between MHS and NR is in the order of some 25 per cent (Bianchi, *publ.*). It thus appears as if MHS also display an increased resistance along more proximal arteries as well as along the true resistance vessels, since the difference in pressure between the carotid and tail arteries is considerably smaller in NR than in MHS.

Nevertheless irrespective of such divergences in mean pressure level along the arterial tree, MHS hypertension seems to be more mild than in SHR and to involve some genetically inherited renal abnormality (Blanchi, Fox and Imbasciani 1973; Bianchi *et al.* 1975 a, b). The hemodynamic consequences of such a renal abnormality which tends to cause an increased plasma volume, have been thoroughly discussed by e.g. Ledingham and Cohen (1969), Guyton and Coleman (1969), Bianchi, Tenconi and Lucca (1970) and Ledingham *et al.* (1971). It has been suggested, that a primary overfilling of the circulatory system leads to increased cardiac filling pressure, and hence to an increased stroke volume and cardiac output. In response to the increased output "whole body auto-regulation" is assumed to take place, upon which cardiac output and blood volume return towards normal (Guyton and Coleman 1971).

It results from the present hemodynamic investigation in MHS rats in general terms, that the above-mentioned sequence of events. Thus, the increased stroke volume found in MHS, in combination with signs of a reduced sympathetic influence on the heart, suggest that a raised cardiac filling pressure was present. The increased stroke volume may explain, at least in part, why systolic pressures in MHS differ particularly much from those in NR (Blanchi *et al.* 1975 a, b), while the differences in mean arterial pressure were more modest.

In the present study. However, despite the increased stroke volume, cardiac output was not increased in MHS. This was most likely due to a reflexogenic slowing of the heart, as judged by the considerably lower heart rate in MHS than in NR. This cardiac slowing in MHS seems primarily to be due to an increase in vagal activity and reduction sympathetic drive, as judged by the relative effects of atropine and propranolol in MHS and NR. Such an increased reflexogenic inhibition of heart rate may at least in part be elicited by ventricular and arterial receptors in response to the relatively large stroke volume and pulse amplitude in MHS. Following complete blockade of cardiac nervous control heart rate was essentially the same in MHS and NR.

Total peripheral resistance was increased in MHS. A "whole body autoregulation" as described above may theoretically account for such a resistance increase, although, of course, other mechanisms may well contribute or may even be more important. It may be said that a chronic type of truly "whole body autoregulation" is constituted by the widespread structural increase of wall/lumen ratio that occurs throughout the systemic precapillary resistance vessels in any type of hypertension (*cf.* Folkow *et al.* 1973). This "structural autoregulation" has in various ways been demonstrated to occur rapidly as a local adaptive process in response to even a few weeks of average increase in pressure load (Folkow *et al.* 1973; Lundgren 1974; Weiss 1974; Hallbäck 1975).

In MHS both the degree of hypertension and the extent of structural vascular adaptation, as judged by the degree of left ventricular weight increase, are decidedly less than in SHR. No analysis of the resistance vessel design has been performed in MHS so far. However, a mild degree of "structural autoregulation" is likely to be present which may at least partly explain the rise in systemic resistance in this type of genetic hypertension. The reason is that the degree of left ventricular hypertrophy and the degree of structural adaptation of the resistance vessel tend in general to run closely parallel when the average blood pressure becomes elevated (Lundgren 1974). In MHS left ventricular hypertrophy was evident as a 15 per cent increase over NR, as judged from the ratio left ventricular weight/body weight.

The present findings thus show that the stroke volume is somewhat increased in MHS, by which these rats differ substantially from other types of spontaneous hypertensive rats, such as the SHR. Thus, in contrast to MHS, when SHR are in their established phase of hypertension, their stroke volume is rather lower than in their controls (Pfeffer and Frenkel 1973). Furthermore, in MHS hypertension, which primarily seems to involve some genetically linked alteration in renal function (Bianchi *et al.* 1975 a, b), the present study has shown that there is no sign of any central neurohormonal "hyper-reactivity" of the type met with in SHR (Hallbäck and Folkow 1974). Thus, unlike SHR, MHS did not exhibit any more pronounced cardiovascular responses to graded stress stimuli as compared with their normotensive controls. Furthermore, they did not show any evidence for an enhanced neurogenic "drive" to the cardiovascular system during "rest". If anything, neurogenic cardiovascular influences in MHS rather appear to act in a manner such as to "dampen" the hypertensive state, as judged from the effects of selective nervous blockade of the baroreceptors.

The primary renal imbalance in MHS involves some fluid and salt retention in early life, causing a relative volume "overload" (Bianchi *et al.* 1975 a, b) which at least initially tends to cause an increased cardiac output in these early phases of life.

over it appears, from the present findings on mature MHS in the established phase hypertension, as if cardiac performance is then reflexly damped resulting in a lower heart rate stroke volume remains somewhat raised. Further analyses of the degree of structural changes and of cardiac receptor and baroreceptor function are required to clarify the complex hemodynamic situation in MHS. However there is no doubt that this of primary genetic hypertension in rats differs from that in SHR or in GHR in several most respects.

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The primary renal imbalance in MHS involves some fluid and salt retention in early life, causing a relative volume "overload" (Bianchi *et al* 1975 a, b) which at least initially tends to cause an increased cardiac output in these early phases, rising arterial

Conjugated Catechol Derivatives in a Transplantable Islet Cell Tumour of the Golden Hamster

By

B. FALCK, C. HANSSON, B. M. KENNEDY and E. ROSENQVIST

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Abstract

FALCK, B., C. HANSSON, B. M. KENNEDY and E. ROSENQVIST. *Conjugated catechol derivatives in transplantable islet cell tumour of the golden hamster*. Acta physiol. scand. 1977 99 217-224.

β-glucuronidated catechol derivatives have been identified in transplantable islet cell tumour of the golden hamster. Dopamine-4-O-glucuronide and 3-methoxytyramine-4-O-glucuronide. L-dopa is rapidly metabolized in the tumour to one or both of these glucuronides. Incubation of tumour homogenates in the presence of β-glucuronidase shows that dopamine-4-O-glucuronide is present in the tumour in extremely high concentrations.

Transplantable, insulin-producing islet cell tumour originally described by Kirman (1962) has been found to contain dopa, dopamine and 5-hydroxytryptamine (Cegrell *et al.* 1969 a) as well as enzymes involved in the synthesis and break-down of monoamines (Cegrell *et al.* 1969 b, Aschman *et al.* 1970). Cegrell *et al.* (1969 a) have also noted the presence of a substance which is fluorogenic in the Falck-Hillarp formaldehyde method (Falck 1962, Falck *et al.* 1962), like the monoamines and their immediate amino acid precursors, but which could not be visualized on paper chromatograms with $K_3Fe(CN)_6$. In this study two substances corresponding to this earlier observation were isolated from the tumour and identified.

Material and Methods

Islet cell tumour tissue was transplanted subcutaneously to adult golden hamsters and allowed to grow for 4-6 weeks. The tumours then weighed 4-10 g and showed no macroscopic signs of necrosis.

In some experiments tumour tissue was homogenized in 0.4 M ice-cold perchloric acid, and the supernatant obtained on centrifugation was purified on Dows 50 W-X4, 200-400 mesh, cation exchange column (3.4 mm), equilibrated with 1 M sodium acetate buffer, pH 6.0. The elution was performed with 1 M HCl. The eluate was collected in two portions of 8 ml each. The unknown substances were identified by transforming them to fluorophores with formaldehyde and hydrogen peroxide at pH 1 according to the tryptamine method of Hess and Udenfriend (1959). For paper chromatography the eluates were evaporated

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since dopa injections increased, and dopa decarboxylase inhibition decreased, the reaction intermediates produced by these 2 substances. Thus, it seemed reasonable to indicate the hypothesis that the 2 compounds were substituted catechol derivatives.

6 tumours were homogenized separately in 6 M HCl (20 ml) and centrifuged for 10 min (7000 rpm). The supernatants were either refluxed for 3 h (3 tumours) or stored at -4°C for 3 h. The acid was evaporated and the residue was dissolved in 0.4 M perchloric acid (1 ml) and adsorbed onto Al_2O_3 ; a dopamine determination was made according to Anton and Seyre (1964). After refluxing, the dopamine content was $10 \pm 1.8 \mu\text{g/g}$ (mean \pm S.D.) now tumour; in the control stored at $+4^{\circ}\text{C}$ the content was significantly less, $3.4 \pm 0.56 \mu\text{g/g}$ ($p < 0.001$).

In another series of expts., 3 tumours were homogenized in 0.4 M perchloric acid and the percolate was passed through the cation resin. The first portions (8 ml) of the eluates were pooled and evaporated to near dryness and the residue was extracted with acetone and stirred. The supernatant was again evaporated to a small volume and divided into 3 equal parts which were applied to a Whatman No. 1 paper. After developing in the same solvent as above, the paper was cut longitudinally into 3 strips. One was treated with formaldehyde gas to localize the two substances with R_f values 0.10 and 0.28. The corresponding rest as well as the areas above and below each spot, i.e. in all 6 areas from each of the remaining strips were extracted with 0.1 M HCl. The 6 extracts from one strip were kept at $+4^{\circ}\text{C}$ for 3 h, and the remaining 6 extracts were refluxed for 3 h in 6 M HCl. In determinations according to Anton and Seyre dopamine could be detected only in the refluxed extract from the spot with R_f value 0.10.

In a third series of expts. focused on the substance with R_f value 0.28, the same procedure was followed up to and including the hydrolysis. The 6 extracts were prepared for gas chromatography-mass spectrometry (GC-MS) by evaporation to dryness, and the formation of derivatives with 40 μl trifluoroacetic acid anhydride in 20 μl ethyl acetate at room temperature for 1/2 h. The solution was then evaporated to dryness and the residue was taken up in 1 μl trifluoroacetic acid anhydride in ethyl acetate. Mass spectra were run on an LKB 2091 spectrometer fitted with a 3% OV 17 column (helium flow rate 20 ml/min column temperature 150°C ion source 270°C ionizing electron energy 70 eV).

Only the derivative from the refluxed extract of the spot gave a significant gas chromatographic peak. The mass spectrum recorded from this peak matched the spectrum recorded from the trifluoroacetoxy derivative of 4-hydroxy-3-methoxyphenylethylamine (Fig. 1). The 2 samples also had the same retention time on gas chromatography. The derivative of 3-hydroxy-4-methoxyphenylethylamine gives a mass spectrum that is significantly different, especially in the intensity of the peak at m/e 233 ($\text{M}-\text{CH}_3\text{NHCOCF}_3$). If the fragment resulting from this decomposition has the structure of a methoxy-trifluoroacetoxybenzyl cation (Tan *et al.* 1962), it should be identical for the 2 isomers. However retention of the benzyl structure could allow resonance stabilization with participation of the electrons on the *p*-methoxy group, giving a stable quinoid structure (Fig. 2). For the *m*-methoxy isomer the strongly electronegative trifluoroacetoxy group in the para-position would not give the same mesomeric effect. The lower stability of the 3-methoxy-4-trifluoroacetoxybenzyl cation should promote further decomposition. This probably occurs with elimination of carbon monoxide.

to near dryness at room temperature and the residue was extracted with acetone. After centrifugation supernatant was evaporated to small volume, applied to paper (Whatman No. 1) and run in $\text{pH} 10.5$ M HCl (9:1). The determination of catechol derivatives was made by the spectrophotofluorometric method of Anton and Sayre (1964) using an Aminco-Bowman spectrophluorometer. Other methodological details will be described below.

L-dopa, dopamine and Type II 2 crude β -glucuronidase solution from *Helix pomatia* (containing a base), were purchased from Sigma (β -glucuronidase activity approx. 100 000 Fishman units/ml; pH activity 1 ml will hydrolyze up to 10 000 μmol of *p*-nitrocatechol sulfate per hr at pH 5 at 37°C). The β -decarboxylase inhibitor NSD 1015 (*m*-hydroxybenzyl hydrazine) was obtained from Smith and Kettlewell.

Results

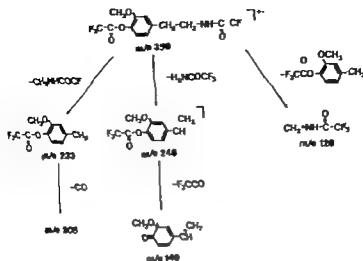
Introductory paper chromatographic and spectrophotofluorometric analyses. In 5 experiments, eluate portions from the cation resin were chromatographed on paper with authentic L-dopa and dopamine. Three chromatograms were exposed to dry formaldehyde gas at 37°C for 1 h (i.e. the same reaction conditions used in the Falck-Hillarp histochemical method for the visualization of certain phenylethyl and indolyethyl derivatives). The first 8 ml in the ion exchange column gave 2 intensely blue fluorescing spots with *R_F* values of 0.10 and 0.28 respectively. The second eluate portion showed one spot with an intense yellow-green fluorescence and the same *R_F* value as authentic dopamine. Two chromatograms were sprayed with 0.44% K Fe(CN)_6 (pH 7.4). Only the dopamine spots appeared.

In a second series of 4 experiments, the material from the first 8 ml of the eluate was divided into 2 equal portions and chromatographed on paper. One half of the paper was treated with formaldehyde gas to localize the 2 blue fluorescent spots. The corresponding areas on the other half of the paper were extracted with 0.1 M HCl and the extracts were treated according to the tryptamine method of Hess and Udenfriend (1959) to produce fluorophores. In both cases a significant fluorescence was obtained with excitation/emission maxima at 350/455 nm (uncorrected instrumental values). These spectral characteristics are clearly different from those displayed by the tryptophan and tryptamine fluorophores which have their excitation/emission peaks at 365/440 nm.

Effect of dopa injections and dopa decarboxylase inhibition. 5 out of 12 tumour-bearing animals were injected intraperitoneally with L-dopa 100 mg/kg b.w. and killed 2 h after the injections. The tumours were removed and extracts prepared as above were purified and treated to produce fluorophores according to Hess and Udenfriend. A comparison of the fluorescence intensities obtained from the 2 groups showed a mean increase by a factor of 2.3 ($p < 0.001$) in the tumours from the animals treated with dopa.

In another series, 11 animals received NSD 1015 100 mg/kg b.w., and the tumours were removed 1.5 h after the injections. 11 tumours from untreated animals served as controls. The dopa decarboxylase inhibition produced a mean decrease in fluorescence intensity by a factor of 0.63 ($p < 0.01$).

Acid hydrolysis. The data above show that the unknown substances have the molecular requirements for condensing with formaldehyde to give intensely fluorescent derivatives in dry milieu (cf. Björklund *et al.* 1972) as well as in solution. They cannot however be visualized on paper with K Fe(CN)_6 in the same way as dopa and the biogenic catecholamines. One or both of the substances seemed to be connected with metabolism in the



Mass spectral fragmentation of 4-hydroxy-3-methoxyphenylethylamine trifluoroacetyl derivative

in 0.1 M sodium acetate buffer (1 g tissue/3 ml buffer) and incubated with Type β -glucuronidase solution. The dopamine content was determined according to Anton *laye* (1964). The results are summarized in Table I. Glucuronic acid was identified according to Kuring and Wegschädel (1971). It is evident from Table I that addition of H-2 extract to the incubation medium results in a pronounced increase of free dopamine. This is presumably due to the β -glucuronidase activity since inhibition of the sulfatase activity of the extract by phosphate ions did not decrease the amount of dopamine formed (columns III and IV in Table I). Incubation without added β -glucuronidase resulted in no formation of dopamine (cf. column I and II in Table I, $p < 0.01$). This would indicate no β -glucuronidase activity in the tumour since the dopa decarboxylase known to be present (Ceprell *et al.* 1969 b), is inactivated at the pH used during incubation. The presence of β -glucuronidase activity in the tumour tissue was established using the

Table I Formation of dopamine from tumour tissues expressed as μ g DA/g tumour tissue (10 ml of homogenate were incubated at +37°C for 1 hr at pH 4.5 for each determination).

I	II	III	IV
Direct extraction with 0.4 M perchloric acid at $\pm 0^\circ$ C	Incubation without added enzymes	Incubation with 1 ml Type H-2 enzyme	Incubation with 1 ml Type H-2 enzyme and 0.1 M sodium phosphate
8.7	16	63	
9.8	11	40	
11.0	17	55	48
8.9	13	43	47
8.5		49	45

β -glucuronidase and sulfatase activity see Material and Methods

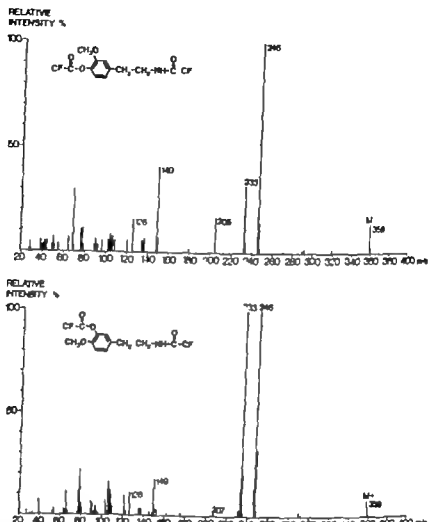


Fig. 1 Mass spectra of trifluoroacetyl derivatives of 4-hydroxy-3-methoxyphenylethylamine and 3-hydroxy-4-methoxyphenylethylamine.

(Costa *et al* 1972) from fragment m/e 233 giving the peak m/e 205 which is not present in the mass spectrum of 4-methoxy-3-trifluoroacetoxy isomer (Fig. 3).

The 2 unknown substances thus yield dopamine and 3-methoxytyramine respectively upon hydrolysis in refluxing acid.

Demonstration of dopamine glucuronide by enzymatic hydrolysis 5 tumours were homo-

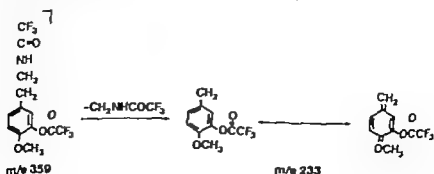


Fig. 2. Mass spectral fragmentation of a resonance stabilized 4-methoxy-3-trifluoroacetoxyphenylethylamine derivative.

tyberyl ester

Discussion

studies using the Falck Hillarp histochemical method, have shown that exposure to ethyl gas gives an intense blue fluorescence in hamster insuloma cells (Cegrell *et al.*

The spectral characteristics were different from those displayed by the fluorophores catechol and indole derivatives which have been studied according to this method in and model systems. This indicated the presence in the insuloma of one or several fixed fluorogenic compounds whose formaldehyde fluorophores had an emission at considerably lower wave lengths than biogenic catechol and indole derivatives so far

In present work, paper chromatographic analyses (phenol -0.1 M HCl 9:1) have separated 2 compounds in the insuloma tissue with Rf values 0.10 and 0.28 respectively develop blue fluorescence when the paper is exposed to formaldehyde gas under the conditions used in the histochemical formaldehyde method.

Hydrolysis of the slow moving compound and of homogenized tumour tissue result formation of dopamine. The substance should thus be a dopamine derivative, possibly a sulfate or the *O*-glucuronide. Incubation of tumour homogenates in the presence of glucuronidase and sulfatase demonstrated considerable formation of dopamine due to glucuronidase activity. The magnitude of this amine formation did not change upon addition of the sulfatase. The compound present in the tumour should thus be a dopamine-glucuronide.

The dopamine-glucuronide could be a mono- or a diglucuronide though from a biological point of view it seems highly improbable that the diglucuronide is formed. To further elucidate the problem it was of importance to compare the properties of the substance in hamster insuloma with those of synthetic dopamine-glucuronide, preferably 4-*O*-(β -D-glucopyranosiduronic acid)-dopamine (DA-4-*O*-glucuronide). To our knowledge a synthesis of this compound has not hitherto been described. A synthetic method had therefore to be worked out (Jonsson and Rosengren 1976). Paper chromatograms showed that the slow-moving substance from tumour extracts had the same Rf value as the synthetic substance and moreover the formaldehyde-induced fluorophores of both substances had the same fluorescence characteristics. Convincing evidence that the dopamine-glucuronide isolated from insuloma tissue is not a diglucuronide but a monoglucuronide substituted in the 4-position was obtained by eluting both this and the synthetic compound from paper chromatograms and testing fluorophore formation with formaldehyde and hydrogen peroxide in solution according to Hess and Udenfriend (1959). The spectral characteristics of the two fluorophores recorded at different pH values revealed a wave-length shift of the excitation maximum and pH dependent fluorescence intensity. These phenomena are significant for fluorophores having a quinoxaline structure implying that they derive from catecholamines with free OH group in the 3-position (*cf.* Jonsson 1966). Thus, DA-4-*O*-glucuronide is present in the hamster insuloma.

The second unidentified substance moved considerably faster on paper chromatograms than the DA-4-*O*-glucuronide. When exposed to formaldehyde gas on paper or when reacted with formaldehyde and hydrogen peroxide according to Hess and Udenfriend (1959)

Sigma kit no 325. Three determinations on single tumours gave 1.325, 1.330, and 1.335 Fishman units per gram tissue.

The enzymatic hydrolysis and direct extraction yielded considerably higher values of dopamine in this experiment than those found with acid hydrolysis and the corresponding control determination. These two experiments can not however be directly compared. Firstly the process involved in the acid hydrolysis has been shown to cause a certain loss of catecholamines. Secondly a contributing reason for this discrepancy may derive from the fact that there have been 18 passages of the tumour between these two studies.

Comparative studies using synthetic 4-O-(β -D-glucopyranosiduronic acid)-dopamine (4-O-glucuronide). The DA-4-O-glucuronide was synthesised (Hansson and Rosengren 1975) and this synthetic compound was used to establish the presence of the substance in insuloma tissue.

Extracts from 3 tumours were run on paper as described above using the synthetic DA-4-O-glucuronide as reference. The spots were visualized using the formaldehyde procedure. In all 3 cases the slow-moving substance from the insuloma had the same R_f value (0.10) and fluorescence characteristics as the reference substance when determined in a spectrophotofluorometer after elution of the fluorophores with 0.1 M HCl. When the synthetic DA-4-O-glucuronide was dissolved in 0.1 M HCl and then treated with formaldehyde and hydrogen peroxide according to Hess and Udenfriend it displayed the same fluorescence characteristics as those of the material isolated from the insuloma. Fluorescence spectra obtained from the 2 substances at pH 1, 7 and 11 were also identical. The emission maxima remained unchanged but a pronounced wave-length shift from 350 nm at pH 1 to 367 nm at pH 7 occurred in the excitation maximum with a concomitant sharp increase in fluorescence intensity. A slight decrease of the excitation maximum and a lower intensity was registered at pH 11.

Demonstration of 3-methoxytyramine and its glucuronide. Tissue extracts were prepared and purified on the cation exchange resin (cf. Material and Methods). 25 ml 1.2 M HCl was passed through the column and the effluent was discarded. Further elution was performed with 20 ml 2 M HCl and the whole eluate was evaporated to dryness. The residue was prepared for GC/MS as above. The mass spectrum revealed the presence of 3-methoxytyramine (Fig. 1).

Extracts from insulomas were run on paper and the fast-moving spot (R_f 0.28) was eluted. The eluate was incubated with Type H-2 crude extract at 37°C and \pm 0°C respectively. Incubates were purified and prepared for GC/MS as described for 3-methoxytyramine. GC/MS analyses showed the presence of this substance only in the extract from the incubation at +37°C.

To get an estimate of the amount of 3-methoxytyramine-4-O glucuronide present in the insuloma, 2 tumours were homogenized in buffer for direct extraction and for incubation in the presence of Type H-2 extract with and without phosphate ions following the procedure described above for dopamine glucuronide. The content of 3-methoxytyramine was determined according to Carlsson and Waldeck (1964). Direct extraction gave no significant amount of 3-methoxytyramine according to this fluorometric method whereas the enzymatic hydrolysis gave 7.6 and 7.2 μ g 3-methoxytyramine/g tumour tissue.

Discussion

For studies using the Falck Hillarp histochemical method, have shown that exposure to formaldehyde gas gives an intense blue fluorescence in hamster insulinoma cells (Cegrell *et al.* 1974). The spectral characteristics were different from those displayed by the fluorophores of catechol and indole derivatives which have been studied according to this method in actual and model systems. This indicated the presence in the insulinoma of one or several unidentified fluorogenic compounds whose formaldehyde fluorophores had an emission peak at considerably lower wave lengths than biogenic catechol and indole derivatives studied so far.

In the present work, paper chromatographic analyses (phenol - 0.1 M HCl, 9:1) have demonstrated 2 compounds in the insulinoma tissue with *R_F* values 0.10 and 0.28 respectively. They develop a blue fluorescence when the paper is exposed to formaldehyde gas under the reaction conditions used in the histochemical formaldehyde method.

Acid hydrolysis of the slow moving compound and of homogenized tumour tissue result in the formation of dopamine. The substance should thus be a dopamine derivative, possibly a *O*-sulfate or the *O*-glucuronide. Incubation of tumour homogenates in the presence of glucuronidase and sulfatase demonstrated considerable formation of dopamine due to β -glucuronidase activity. The magnitude of this amine formation did not change upon addition of the sulfatase. The compound present in the tumour should thus be a dopamine glucuronide.

This dopamine-glucuronide could be a mono- or a diglucuronide though from a biological point of view it seems highly improbable that the diglucuronide is formed. To further elucidate this problem it was of importance to compare the properties of the substance in hamster insulinoma with those of synthetic dopamine-glucuronide, preferably 4-*O*-(β -D-glucopyranosyluronic acid)-dopamine, (DA-4-*O*-glucuronide). To our knowledge a synthesis of this compound has not hitherto been described. A synthetic method had therefore to be worked out (Hansson and Rosengren 1976). Paper chromatograms showed that the slow-moving substance from tumour extracts had the same *R_F* value as the synthetic substance and moreover that the formaldehyde-induced fluorophores of both substances had the same fluorescence characteristics. Convincing evidence that the dopamine-glucuronide isolated from insulinoma tissue is not diglucuronide but monoglucuronide substituted in the 4-position was obtained by lifting both this and the synthetic compound from paper chromatograms and inducing fluorophore formation with formaldehyde and hydrogen peroxide in solution according to Hess and Udenfriend (1959). The spectral characteristics of the two fluorophores recorded at different pH values revealed a wave-length shift of the excitation maximum and a pH dependent fluorescence intensity. These phenomena are significant for fluorophores having a quinoidal structure implying that they derive from catecholamines with a free OH-group in the 3-position (*cf.* Jonsson 1966). Thus, DA-4-*O*-glucuronide is present in the hamster insulinoma.

The second unidentified substance moved considerably faster on paper chromatograms than the DA-4-*O*-glucuronide. When exposed to formaldehyde gas on paper or when treated with formaldehyde and hydrogen peroxide according to Hess and Udenfriend (1959)

It displayed the same fluorescence characteristics as the DA-4-O-glucuronide at pH 7.5 and yielded no dopamine upon acid hydrolysis. Since the occurrence of 3-methoxytyramine in the tumours was established in the present work, it appeared reasonable to assume the substance could be 3-methoxytyramine-4-O-glucuronide. In fact incubation with β -glucuronidase or acid hydrolysis resulted in the formation of a substance that in GC-MS was unequivocally identified as 3-methoxytyramine.

Thus, there are at least two conjugated catechol derivatives in this islet cell tumour. One of these, the dopamine-glucuronide, is present in extremely high concentration, greater than hitherto found in any mammalian tissue. Both can be estimated by the Hess and Ué (1959) fluorescence reaction but this does not distinguish between them. Administration of L-dopa or the dopa-decarboxylase inhibitor NSD 1015 produces, within 2 h or less, considerable changes in the concentrations of these two substances as measured by the fluorescence reaction. There is thus a rapid turnover of L-dopa and formation of one or more of the glucuronides in the tumour tissue.

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Actomyosin ATPase, Myokinase, CPK and LDH in Human Fast and Slow Twitch Muscle Fibres

By

ALL THORSTENSSON, BERTE SJÖÖRN, PER TERCH and JAN KARLSSON

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Abstract

THORSTENSSON, A., B. SJÖÖRN, P. TERCH and J. KARLSSON. *Actomyosin ATPase, myokinase, CPK and LDH in human fast and slow twitch muscle fibres*. Acta physiol. scand. 1977 99 225-229.

The activities of Mg^{2+} stimulated ATPase, creatine phosphokinase (CPK), myokinase (MK) and lactate dehydrogenase (LDH) were determined in pooled fast twitch (FT) and slow twitch (ST) human skeletal muscle fibres, dissected out from freeze-dried muscle biopsy material. All enzymes investigated demonstrated higher activities in FT fibres. The ratio in enzyme activity between fibre types was greatest for Mg^{2+} stimulated ATPase (2.1) and smallest for CPK (1.3). In addition, the isoenzyme patterns of CPK, MK and LDH were studied by means of isoelectric focusing (CPK and MK) and disc electrophoresis (LDH). A difference was observed between fibre types with respect to the isoenzyme distribution of MK and LDH, whereas the CPK isoenzyme pattern was similar in both fibre types. These results on separated human FT and ST fibres are essentially in conformity with what has earlier been indicated from experiments on mixed muscle homogenates.

Most human skeletal muscles contain a mixture of fast twitch (FT) and slow twitch (ST) muscle fibres. The relative distribution of these two main fibre types may however vary considerably (Gollnick *et al.* 1972 and 1974). By biochemical analyses of muscle homogenates with a wide range of fibre compositions it has been possible to demonstrate muscle fibre dependency of several enzymes involved in the energy yielding processes of the muscle (Gollnick *et al.* 1974, Essen *et al.* 1975).

Such studies have formed the basis for the metabolic profiles assigned the two main human muscle fibre types, a high glycolytic capacity for FT fibres and a high oxidative potential for ST fibres. Also some of the enzymes involved in the immediate ATP turn-over in connection with muscle contraction have been demonstrated to have activities that varied with muscle fibre composition. Thus, muscle samples with a high proportion FT fibres showed a higher activity of myosin ATPase (Taylor *et al.* 1974), Mg^{2+} stimulated ATPase, and myokinase (MK) (Thorstenson *et al.* 1976 a). Furthermore, in the case of enzymes like lactate dehydrogenase (LDH) and MK, muscle fibre type related differences have been observed also in isoenzyme distribution (Karlsson *et al.* 1974, Thorstenson *et al.* 1976 b).

Similar relationships have been reported for creatine phosphokinase (CPK) in muscle (Sherwin *et al.* 1967) but could not be confirmed in mixed human muscle homogenates (Thorstensson *et al.* 1976 a and b).

Recently however a technique has been described to dissect out and identify FT and ST fibres from freeze-dried muscle biopsy material (Essén *et al.* 1975). This makes it possible to perform biochemical analyses on pure samples of the two fibre types in human skeletal muscle: FT and ST fibres.

The purpose of the present investigation was to use this technique to compare the isoenzyme patterns of Mg²⁺ stimulated ATPase, CPK, MK, and LDH in FT and ST muscle fibres and thereby examine the validity of results obtained in experiments on mixed muscle homogenates.

Material and methods

Material. Muscle biopsies were obtained at rest with the needle biopsy technique (Bergström 1962) vastus lateralis of two habitually active healthy male subjects (21 and 25 yrs of age).

Muscle fibre preparation. The method for preparation of single muscle fibres was essentially as described by Essén *et al.* (1975). Briefly dissection of fibres was performed from freeze-dried material in a section microscope in a room with constant temperature and humidity (20°C and 35% RH). Fibre classification as FT or ST was done on the cut off ends of each fibre, which were stained for fibrillar ATPase (Padykula and Herma 1955) for preincubation at pH 10.3. The remaining parts of the fibres were pooled together according to fibre type. The total number of fibres dissected was 1818 FT and 1804 ST fibres, respectively. Before further analyses the pooled muscle fibres were homogenized by sonication (200/1 0.5 M KCl).

Biochemical analyses. The enzyme activities were determined fluorometrically using NADH coupled reactions according to Lowry (Lowry and Passonneau 1972). The enzymes under investigation were Mg²⁺ stimulated ATPase (E.C. 3.6.1.4), creatine phosphokinase, CPK (E.C. 2.7.3.2), or myokinase, MK (E.C. 2.7.4.3), and lactate dehydrogenase, LDH (E.C. 1.1.1.27). The test procedures were as those earlier used for the corresponding enzyme activity analyses on whole muscle homogenates. Methodological errors for these determinations were found to be 8% (Mg²⁺ stimulated ATPase), 10% (CPK and MK), respectively (Thorstensson unpublished results). The effect of freeze-drying on the activities of these enzymes was investigated in a separate study. No decrease in enzyme activities was found in freeze-drying procedures. The dry weight corresponded to 3.5% of the wet weight of the samples.

The protein content of the homogenized fibres was determined with the folin reaction according to Lowry (Lowry *et al.* 1951).

Isozyme separation. The techniques for isoenzyme analysis were the same as earlier described for muscle homogenates (Karlsson *et al.* 1974, Thorstensson *et al.* 1976 b). LDH isoenzymes were separated on entional disc electrophoresis, whereas isoelectric focusing in polyacrylamide and gradient gel electrophoresis were used for CPK and MK isoenzyme separation, respectively. The isoenzymes were detected by zymogram techniques and the relative intensity of the stained band was evaluated by densitometry. (For detailed information, see Karlsson *et al.* 1974, Björn *et al.* 1976, and Thorstensson *et al.* 1976).

Results and discussion

Enzyme activities. All enzymes investigated demonstrated higher levels of activity in FT than in the ST fibres (Table I).

The greatest difference was seen for Mg²⁺ stimulated ATPase. The activity of this enzyme, which is regarded to be controlling the contractile activity in muscle (Perry 1976), was about 3 times higher in the FT fibres. A similar relationship was found for myokinase (MK) from experiments on mixed muscle homogenates.

Table 1. Enzyme activities in pooled fast-twitch (FT) and slow-twitch (ST) skeletal muscle fibres dissected out from freeze-dried human biopsy material.

type	FT	ST	FT/ST ratio
ATPase	0.84	0.30	2.8:1
ATPase:phosphocreatine	16.6	13.1	1.3:1
ATPase	12.1	6.6	1.8:1
Pyruvate:pyruvate	3.66	1.45	2.5:1
Pyruvate:pyruvate	3.66	1.80	2.0:1

Units are in units $\cdot 10^{-4}$ g protein $^{-1}$ min $^{-1}$. LDH is short for lactate dehydrogenase.

mixed muscle homogenates, although not until after a period of physical conditioning (Thorstenson *et al.* 1976). The ATPase activity of purified human myosin has been shown to be 2-3 times higher in FT muscle fibres (Essén *et al.* 1975). The finding was also in conformity with results obtained from animal skeletal muscle preparations, where a high proportion dark staining (FT) fibres in the muscle coincided with a high actomyosin ATPase activity (Griffith and Semmler 1969).

The MK activity in FT fibres was about twice that in ST fibres. This ratio between fibres in MK activity was somewhat lower than could be obtained by extrapolating the tension lines of earlier obtained correlations between % FT and MK activity (Thorstenson *et al.* 1976 a, Korvi *et al.* to be published). However, the general tendency of a higher MK activity in FT muscle fibres found in those studies was confirmed.

A higher activity of CPK in FT fibres has been indicated in animal experiments (Burke and Edgerton 1975). A similar relationship for CPK was not found in human skeletal muscle (Thorstenson *et al.* 1976 a). In the present study the difference in CPK activity between FT and ST fibres was relatively small, about 30%, which could explain the difficulty to obtain any correlation between enzyme activity and muscle fibre type in the above mentioned experiments on mixed human muscle homogenates.

LDH activity was higher by a factor of 2-2.5 in FT as compared to ST muscle fibres irrespective in which direction the enzyme activity was determined. This was in agreement with earlier observations in human skeletal muscle (for refs. see Sjödin 1976). Moreover, it could be calculated that the LDH activity in the forward direction (pyruvate \rightarrow lactate) or the backward direction (lactate \rightarrow pyruvate) was about the same in both fibre types (6.1 and 1.9 in FT and ST fibres, respectively).

Iszyme distribution. The iszyme pattern of CPK after isoelectric focusing showed no difference between FT and ST muscle fibres. In both cases 2 major bands were visible with isoelectric points (pIs) of about 7.4 and 7.0, respectively. The most alkaline band constituted in both instances approximately 70% of total stain density. These figures were in good accordance with values earlier obtained for mixed muscle homogenates (Thorstenson *et al.* 1976 b).

Also the separation of MK isozymes gave results in agreement with what had previously been postulated from whole biopsy analysis (Thorstenson *et al.* 1976 b). Thus, two MK bands were observed in the alkaline region of the gel with pIs of approximately 10.0 (MK 1) and 9.0 (MK 2), respectively (Fig. 1). The MK 1 band predominated in both fibre types,

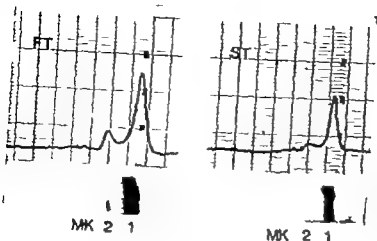


Fig. 1. The isoenzyme pattern myokinase (MK) in pooled fast twitch (FT) and slow twitch (ST) muscle fibres after polyacrylamide gel electrophoresis. The stained gels and the corresponding densitometric scanning are shown.

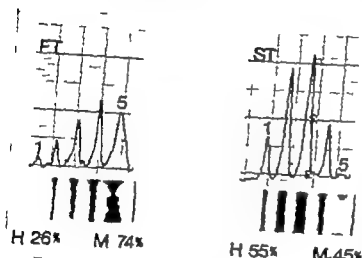


Fig. 2. The isoenzyme pattern lactate dehydrogenase (LDH) in pooled human fast twitch (FT) and slow twitch (ST) muscle fibres after disc electrophoresis. The results of densitometric scanning are shown as well as the calculated portion of H (heart) and M (muscle) type of LDH isoenzymes.

but the relative contribution of this isoenzyme to total stain density was higher in ST fibres (57%) as compared to FT fibres (26%). The faint MK 2 band in ST fibres was hardly visible upon reproduction (Fig. 1).

The LDH isoenzyme pattern demonstrated that the skeletal muscle specific subunit, the M subunit (for refs. see Sjödin 1976) predominated in the FT fibres, whereas the heart muscle specific subunit, the H subunit, appeared with a higher relative stain density in the ST fibres (Fig. 2). These findings confirmed observations earlier made by Sjödin (1976) and would support the suggestion of a more pronounced anaerobic glycolytic potential in the FT fibres, and a higher capacity for lactate combustion in the ST fibres.

In summary the present results obtained on pooled single muscle fibres essentially confirmed what had earlier been deduced from experiments on mixed human skeletal muscle homogenates. Thus, the conclusions made in those studies about a metabolic profile of FT muscle fibres that is more in favour of fast contraction and rapid ATP replenishment as compared to ST muscle fibres were further strengthened.

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Lactate in Fast and Slow Twitch Skeletal Muscle Fibres of Man during Isometric Contraction

By

PER TESCH and JAN KARLSSON

Received 9 August 1976

Abstract

TESCH P and J KARLSSON *Lactate in fast and slow twitch skeletal muscle fibre during isometric contraction* Acta physiol scand. 1977 99 230-236.

Concentrations of lactate in fast and slow twitch fibres, respectively were determined in m. femoris after sustained contractions at 25%, 50%, and 75% of maximal voluntary isometric (MVC) until exhaustion as well as after interrupted exercises at 25 and 50 MVC. Maximal concentrations were only found at 50% of MVC performed to exhaustion. Lactate concentrations higher in slow twitch (ST) fibres at 50% MVC compared to m. ST fibres at 25 MVC, and fast twitch (FT) fibres at 50% MVC compared to FT fibres at 75% MVC. After short time isometric (i.e. 75% to exhaustion and 50% and 25% performed for the same period of time as 75% MVC concentration, expressed as lactate ratio (lactate concentration in FT fibres/lactate concentration in ST fibres) was found to be positively correlated to percent FT fibres ($r=0.89$). Lactate ratio range 1.0-1.8. At onset of isometric exercise, lactate concentration increase was faster in ST fibres in the m. ST fibres and faster in FT fibres when the muscle was rich in FT fibres.

With exhaustive isometric exercise maximal values for ATP and creatine phosphorylation as well as lactate accumulation are observed at tensions corresponding to 30-40% maximal voluntary contraction (MVC) (Karlsson and Ollander 1972, Karlsson *et al.* 1973). Similar high values are obtained during short-term maximal dynamic (bicycle) work (Karlsson and Saltin 1970, Karlsson 1971). At lower (10-25% MVC) and higher (75% MVC) exhaustive isometric tensions "submaximal" values for phosphagen depletion and lactate accumulation are observed (Karlsson and Ollander 1972). At tensions less than 20% MVC mainly slow twitch (ST) fibres are depleted on their glycogen (Gollnick *et al.* 1974a) according to estimation of glycogen content in individual muscle fibres, on the basis of PAS intensity (Gollnick *et al.* 1972), whereas mainly fast twitch (FT) fibres are depleted on their glycogen at tensions in excess of 25% MVC. If the selective glycogen depletion represents muscle fibre recruitment it would indicate that at light tensions only ST fibres are recruited, while at heavier tensions only FT fibres are recruited. Gydikov (1975) has reported that at light (30-40% MVC) isometric muscle tensions only slow motor units are active. First at tensions in excess of 30-40% MVC fast motor units are recruited but slow motor units are still discharging.

Table 1. Individual anthropological and physiological data.

Subject	Age (yr)	Height (cm)	Weight (kg)	Fibre comp (% FT)	MVC (kp)	Max. performance time, min MVC		
						75	50	25
25	179	71	68	202	0.46	0.94	3.40	
21	189	83	58	198	0.70	1.42	5.90	
19	185	76	45	246	0.90	1.16	4.00	

As great variations in lactate accumulation are present within a whole biopsy specimen (Jansson 1971) it seems reasonable to suggest that exhaustion at low as well as high tensions are depending on high lactate concentrations and phosphagen depletions in recruited motor units. The present study was therefore undertaken to evaluate lactate concentration in individual muscle fibre types at different isometric tensions performed either submaximally or to exhaustion.

Subjects, procedure and methods

Healthy male students of physical education are employed as subjects. Some of their characteristics are listed in Table 1. Isometric exercise was performed on a chair constructed for leg exercise (Karlsson and Olsson 1972). By pressing the feet on a sitting position, against an immovable bar mainly the thigh muscles are activated (Karlsson *et al.* 1975). The subjects trained in the chair 2-3 days prior to the experiments, learn the technique used to get accustomed with the experimental set up. The day before the experiments, maximal voluntary contraction (MVC) was determined. The final experiments were conducted over two days. 75, 50, and 25 % of MVC were calculated for each subject and performed to exhaustion. The rest period between the exercise bouts was at least 2 h. In the second set of experiments, the subjects were loaded at 50 and 25 % of MVC separately but the experiments were interrupted at the time corresponding to exhaustion for 75 % MVC. In Table 1 the protocol of the experiments is illustrated.

EXPERIMENTS

MUSCLE WORK TO EXHAUSTION

FIRST BOUT

SECOND BOUT

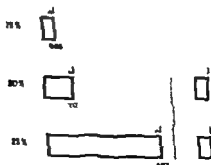


Fig. 1. Experimental protocol. Mean time (min) are expressed below the squares.

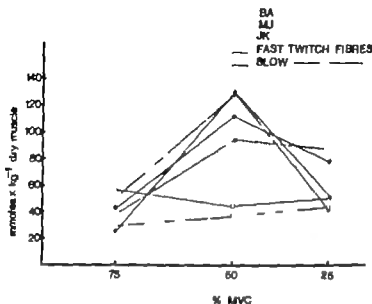


Fig. 2. Lactate concentration in fast twitch (FT) and slow (ST) muscle fibres, resp. after exhaustive 75, 50, 25% MVC leg muscle contraction.

Muscle biopsies (Bergström 1962) were taken from the lateral portion of the quadriceps femoris before the exercise tests for determination of muscle fibre composition according to Gollnick *et al.* Immediately after termination of exercise (within 3–5 s) with the subject still sitting in the chair, biopsies were taken for determination of muscle fibre lactate concentration. The specimens were immediately frozen in liquid nitrogen and stored at -80°C for later analysis (Karlsson 1971).

Analysis for lactate concentration on individual muscle fibre groups were made on muscle fibres from freeze dried biopsies (Esmén *et al.* 1975). The freeze dried biopsy specimens were then in vacuum at -80°C before analysis. After dissecting at 21°C with a humidity of 30%, the fibres identified by means of myofibrillar ATPase staining (for ref. see Esmén *et al.* 1975). Identified ST and FT fibres, respectively, were then pooled and weighed. Fluorometric techniques (Karlsson 1971) have been used to determine lactate concentration in 30–60 slow and fast twitch fibres, respectively, in each sample. The weight of the fibres independent of fibre type, were in each determination between 150 μg . When analysing fibres from the same freeze dried biopsy specimen at separate days (week) the coefficient of variation was 14% as compared to a coefficient of variation of 6% between different parts of the biopsy the same day.

Results

The pattern of maximal endurance time in relation to relative force was similar to what has been reported (Monod and Sherrer 1957, Rohmert 1960, Humphreys *et al.* 1963, Karlsson and Ollander 1972, and Karlsson *et al.* 1975). The mean performance at 75, 50 and 25% MVC, respectively, averaged 0.55 (range 0.46–0.70) min, 1.17 (0.94–1.42) min and 4.63 (range 3.40–5.90) min.

With exhaustive exercise mean lactate concentration was highest after 50% MVC in ST as well as FT fibres, 86 (range 37–128) and 96 (range 44–130) mmoles kg^{-1} dry weight, respectively. This was true for 2 of 3 subjects studied. One subject demonstrated approximately the same lactate concentration after exhaustive 75% and 25% MVC, respectively. 75 and 25% MVC resulted in a mean lactate concentration of 41 (FT fibres) and 41 (ST fibres), and 60 (FT fibres) and 58 (ST fibres) mmoles kg^{-1} dry weight, respectively. Individual values are presented in Fig. 2. The values after 50% MVC should be expressed as

ISOMETRIC SHORT TIME EXERCISE

BA	0.48
MJ	0.70
JK	0.60

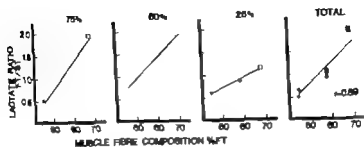


Fig. 3. Lactate ratio, each means lactate concentrations in FT fibres versus lactate concentrations in ST fibres, expressed in relation to muscle fibre composition (% FT) after 75, 50 and 25% MVC performed for one period of time at 75% MVC to exhaustion, $p < 0.001$, $y = 0.04 - 1.34$.

wet weight, reach about 20–22 (Karlsson 1971). Lactate concentration obtained at exhaustion for the 3 different tensions are in close agreement with those earlier obtained from biopsy specimens (Karlsson and Ollander 1972, and Karlsson *et al.* 1975). For "submaximal" 50% and 25% MVC lactate concentration averaged 35 (FT fibres), 32 (ST fibres), and 29 (FT fibres), and 41 (ST fibres) mmol kg^{-1} dry weight. These concentrations were consequently similar to the values obtained at 75% MVC and exhaustion, indicating a similar rate of initial muscle fibre lactate accumulation irrespective 25–50–75% MVC.

Muscle fibre composition for the 3 subjects varied (Table I). Thus one subject (BA) had 48% FT fibres and another (JK) 45% FT fibres. If lactate content was expressed as lactate concentration in FT fibres versus ST fibres (lactate FT/ST ratio, Fig. 3) subject BA had a ratio of 1.93 after exhaustive short time exercise (75% MVC) while the corresponding ratio for subject JK reached 0.51. After "submaximal" (50% MVC) short time exercise lactate ratio for subject BA was 1.99 and for subject JK 0.88. For subject MJ (58% FT fibres) the corresponding values were 1.07 and 1.02, respectively. After "submaximal" (25% MVC) short time exercise the lactate ratio for BA, MJ and JK were 1.13, 0.83 and 0.63, respectively. When the 50 and 25% MVC tensions were maintained until exhaustion, the lactate FT/ST ratio approached unity for the two extremes BA and JK, respectively (Fig. 4).

Discussion

Lactate concentration differences between muscle and blood in man immediately after short time (6–10 min) submaximal or maximal bicycle exercise suggest that a fast translocation of lactate does not take place neither between lactate producing fibres and the extracellular space nor the extracellular space and nonrecruited fibres (Karlsson 1971 and Jorfeldt *et al.* 1974). In fact after short time maximal exercise 6–10 min has to lapse before an equilibrium is

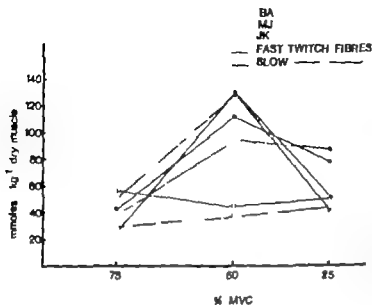


Fig. 2. Lactate concentration fast twitch (FT) and slow twitch (ST) muscle fibres, respectively at exhaustion after 75, 50 and 25% MVC leg muscle contraction.

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Analysis for lactate concentration on individual muscle fibre groups were made on muscle fibre biopsies from freeze dried biopsies (Esaño *et al.* 1975). The freeze dried biopsy specimens were then placed in vacuum at -80°C before analysis. After dissecting at 1°C with a humidity of 30%, the fibres identified by means of royal brilliant ATPase staining (for ref. see Esaño *et al.* 1975). Identified ST like FT fibres, respectively were then pooled and weighed. Fluorometric techniques (Karlsson 1971) have been used to determine lactate concentration in 30–60 slow and fast twitch fibres, respectively per each sample. The weight of the fibres independent of fibre type, were in each determination between 150 μg . When analysing fibres from the same freeze dried biopsy specimens at separate days (at least one week) the coefficient of variation was 14% as compared to coefficient of variation of 6% between different parts of the biopsy the same day.

Results

The pattern of maximal endurance time in relation to relative force was similar to earlier has been reported (Monod and Sherrer 1957, Rohmert 1960, Humphreys and 1963, Karlsson and Ollander 1972, and Karlsson *et al.* 1975). The mean performance at 75, 50 and 25% MVC, respectively averaged 0.55 (range 0.46–0.70) min, 1.17 (0.94–1.42) min and 4.63 (range 3.40–5.90) min.

With exhaustive exercise mean lactate concentration was highest after 50% MVC ST as well as FT fibres, 86 (range 37–128) and 96 (range 44–130) mmoles kg^{-1} dry weight, respectively. This was true for 2 of 3 subjects studied. One subject demonstrated approximately the same lactate concentration after exhaustive 75% and 25% MVC, respectively. The same lactate concentration after exhaustive 75% and 25% MVC resulted in a mean lactate concentration of 41 (FT fibres) and 46 (ST fibres), and 60 (FT fibres) and 58 (ST fibres) mmoles kg^{-1} dry weight, respectively. Individual values are presented in Fig. 2. The values after 50% MVC should, expressed as mean

been suggested that at short time maximal exercise (below 1 min) neuromuscular fatigue take place and is most marked in high threshold motor units, while contraction fatigue affects low threshold motor units (Stephens and Taylor 1972). Another alternative for performance time and exhaustion could possibly be the rate of store rise in activated muscle fibres. It was recently pointed out (Bolsland and Eng-76) that heat production was greater in FT fibres compared to ST fibres and that the temperature rise was twice as high in muscles rich in FT fibres (*m. biceps brachii*) pared to muscles rich in ST fibres (*m. soleus*).

subject with a high percent FT fibres had a higher lactate concentration in the FT expressed as lactate ratio FT/ST as compared to the subject with a low percent FT. This observation was made after exercise at different tensions, exhaustive or not, performance time corresponding to about 0.5 min. This means that the subject rich in twitch fibres (JK) in all tensions studied after 0.5 min of exercise demonstrated lactates in the ST fibres (Fig. 3).

FT/ST lactate ratio in the subject with a low percent FT fibres in his muscle seems to dict the conclusion by Gollnick *et al.* 1974 a, that at sustained contractions at low ric tensions there is a major reliance upon ST fibres and above that level a primary lence upon FT fibres. It is not at present possible to causally explain this difference. fact of individual variation in muscle fibre populations was, however not considered (ick *et al.* 1974 a, as no extremes in that respect were included in the study (Karlsson, com.). It has been reported that subjects with either a high percent ST or a high per ype II A (*Le* FT fibres with a high oxidative potential) do not fit into the glycogen on pattern as suggested by Gollnick *et al.* 1974 a (Komi, pers. comm.).

activity of the enzyme lactate dehydrogenase (LDH) is lower in muscles rich in ST compared to muscles rich in FT fibres (Gollnick *et al.* 1974 b and Sjödin 1976). separating the different LDH isozymes (Karlsson *et al.* 1974) it is evident, that in ion to higher LDH activity the FT fibres contain a higher percent of the LDH isozyme, LD-5 or the most skeletal muscle specific isozyme as compared to ST fibres, which are in LDH 1 or the heart muscle specific isozyme. Moreover it has been suggested (Baba Sharma 1971) that LDH-1 and LDH 2 are localized in mitochondria, which are ind in aerobic metabolism while LDH-5 is found in sarcoplasmic reticulum and should volved in the anaerobic glycolysis.

seems reasonable to assume that similar properties of the different muscle fibre types effect lactate metabolism as indicated by Sjödin (1976). The present results in subject cannot, however be interpreted otherwise than a more marked lactate production was ng place in his ST fibres, which would indicate a recruitment of predominantly ST fibres n tensions studied.

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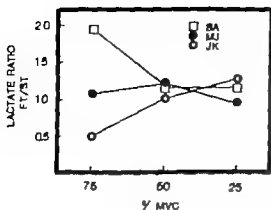


Fig. 4 Lactate ratio (for explanation see Fig. 3). 75, 50 and 25% MVC maintained to exhaustion.

established (for ref. see Karlsson 1971). It seems then reasonable to suggest that in this translocation processes for lactate only to a minor extent might obscure the pattern of lactate accumulation in the muscle related to lactate production. For the same reasons of the extracellular lactate contaminating the freeze dried muscle fibre samples can be neglected as of significance for the interpretation of the results.

The major finding in the present study was that lactate concentration in individual muscle fibres did not correspond to values as could be expected from glycogen depletion pattern in similar experimental situations (Gollnick *et al.* 1974a). High lactate concentrations in FT fibres were not found after exercise corresponding to heavy isometric tensions, and high lactate concentrations were not found in ST fibres after light isometric tensions. Mean lactate concentration was obtained only after isometric, exhaustive exercise at 40% MVC and that was true for lactate concentration in both FT and ST fibres. One subject did however differ from this pattern. He had a muscle fibre composition with a predominance of FT fibres (68%) and also the shortest performance time at all tensions studied. The difference in lactate concentration between the main muscle fibre types was not significant at any tension. Thus, as lactate concentration at exhaustive exercise at 75 and 25% MVC respectively did not reach maximal values in any fibre type, it seems reasonable to suggest that lactate concentration in recruited fibres accordingly cannot explain exhaustion at all tensions.

In the present study 30 or more individual fibres were pooled before analyses. Essen-Häggmark (1976) have demonstrated large variations in lactate content in individual muscle fibres picked from a pool of the same fibre type. This might indicate that exhaustion is primarily not related to the type of muscle fibre recruited but rather which fast or slow motor units are recruited.

If a motor nerve recruited innervates ST fibres then their endurance capacity will be decisive for performance (for ref. see Closs 1972 and Burke and Edgerton 1975). The metabolic profile of the ST fibre population in human skeletal muscle speaks in favour for a pronounced endurance capacity of this main muscle fibre type (Gollnick *et al.* 1974, Karlsson *et al.* 1974, and Essén *et al.* 1975) and this has earlier been confirmed for isometric exercise (Hultén *et al.* 1974) and recently for dynamic exercise (Thorstensson and Karlsson 1976).

Effect of Changes in Blood Volume Distribution on Circulatory Variables and Plasma Renin Activity in Man

By

STURE BEVEGÅRD, JAN CASTENFORS and LAUS ERIK LINDBLAD

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Abstract

Dr S. J. CASTENFORS and L. E. LINDBLAD. *Effects of changes in blood volume distribution on circulatory variables and plasma renin activity in man*. Acta physiol. scand. 1977 99: 237-245.

In six healthy subjects the pressure around the lower body was changed to 40 mmHg above (LBNP) and below (RNP) atmospheric, thereby altering the amount of blood pooled in the lower body. Heart rate, arterial blood pressure, central venous pressure, cardiac output (dye dilution technique) and forearm venous occlusion plethysmography were measured. Plasma renin activity was determined by a radioimmunoassay method. Subjects maintained relative circulatory steady state during LBNP caused significant decreases in central venous pressure (CVP), stroke volume and cardiac output (Q) with an unchanged mean arterial pressure (MAP). Heart rate (HR) increased significantly. Total peripheral vascular resistance (TPVR) and regional vascular resistance (RVR) in the forearm were significantly increased 5-9 min after the onset of LBNP, whereas plasma renin activity (PRA) showed a definite increase only after 19 min of LBNP stimulation. No correlation was found between the changes in PRA and TPVR or RVR. Increasing the pressure around the lower body resulted in a slight but significant increase in MAP as well as a significant but transient increase in Q . No significant changes were found in HR, Q or TPVR. In the forearm, decrease in RVR was found. PRA was not significantly changed. The results demonstrate that peripheral vascular resistance and PRA are both influenced by changes in blood volume distribution, possibly elicited via pressure receptors sensitive to changes in central blood volume and/or CVP. The results also indicate that PRA does not play any significant part in the vasoconstriction during LBNP stimulation.

Key words: Blood volume, pressure receptors, cardiac output, heart rate, vascular resistance, plasma renin activity.

According to the classical concept, changes in the constrictor tone of the resistance vessels are initiated via baroreceptors in the arterial high pressure circulation (Haymans and Neil 1966).

However, it has been observed that even a marked increase, of the order of 40 mmHg in the transmural pressure of the carotid sinus, stimulating a considerable increase in MAP, results in only small changes in RVR (Bevegård and Shepherd 1966, Bevegård, Castenfors and Lindblad, to be published).

On the other hand, during peripheral pooling of blood, causing decreased venous return, there is a marked vasoconstriction, at least in the forearm, before or even in the absence of

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Effect of Changes in Blood Volume Distribution on Circulatory Variables and Plasma Renin Activity in Man

By

STURE BEVEGÅRD, JAN CASTENFORS and LAUR ERIK LINDBLAD

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Abstract

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Eight subjects had the pressure around the lower body increased to 40 mmHg above (LBPP) and (LBNP) atmospheric, thereby altering the amount of blood pooled in the lower body. Heart rate, arterial blood pressure, central venous pressure, cardiac output (dye dilution technique) and forearm flow (venous occlusion plethysmography) were measured. Plasma renin activity was determined radioimmunochemical method. Six subjects maintained relative circulatory steady state during LBNP caused significant decreases in central venous pressure (CVP), stroke volume and cardiac output (Q) at an unchanged mean arterial pressure (MAP). Heart rate (HR) increased significantly. Total peripheral vascular resistance (TPVR) and regional vascular resistance (RVR) in the forearm were significantly increased when measured 5-9 min after the onset of LBNP whereas plasma renin activity (PRA) showed definite increase only after 19 min of LBNP stimulation. No correlation was found between the changes in PRA and TPVR or RVR. Increasing the pressure around the lower body resulted in a slight but significant increase in MAP as well as a significant but transient increase in Q . No significant changes were found in HR, Q or TPVR. In the forearm decrease in RVR was obtained. PRA was not significantly changed. The results demonstrate that peripheral vascular tone and PRA are both influenced by changes in blood volume distribution, possibly elicited via thoracic receptors sensitive to changes in central blood volume and/or CVP. The results also suggest that PRA does not play any significant part in the vasoconstriction during LBNP stimulation.

Keywords: Blood volume, pressure receptors, cardiac output, heart rate, vascular resistance, plasma renin activity.

According to the classical concept, changes in the constrictor tone of the resistance vessels is mediated via baroreceptors in the arterial high pressure circulation (Heymans and Noll 1951).

However it has been observed that even a marked increase, of the order of 40 mmHg the transmural pressure of the carotid sinus, stimulating a considerable increase in MAP results in only small changes in RVR (Bevegård and Shepherd 1966, Bevegård, Castenfors and Lindblad, to be published).

On the other hand, during peripheral pooling of blood, causing decreased venous return, there is marked vasoconstriction, at least in the forearm, before or even in the absence of

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of baroreceptors are stimulated by neck suction. The method for and findings during carotid occlusion are presented in a separate publication (Bevegård *et al.* 1977).

Methods

Blood pressure were measured with strain-gauge transducers (Statham P 23 and Statham PBD 30) fixed on an ultraviolet recorder (ABEM Ultralett). The reference level was the midthoracic level spine position. Mean arterial pressure was obtained by electrical integration.

Stroke output (Q) is determined by dye dilution technique, using indocyanine green as indicator as colorimetrically.

Heart rate (HR) was monitored continuously by a heart rate meter (Xaven Electrocardiograph EQ 11).

Peripheral vascular resistance ($TPVR$) was calculated as the mean blood pressure minus the central pressure divided by the cardiac output and expressed in arbitrary units.

Forearm blood flow (FBF) is measured by venous occlusion plethysmography. The plethysmograph is of an air-filled rubber cuff (Graf and Westerhof 1979). Forearm blood flow was calculated as the slope of three inflow curves.

Renal vascular resistance (RVR) was calculated as the mean arterial pressure divided by forearm blood flow and expressed in arbitrary units.

Plasma renin activity (PRA) is determined with a radioimmunoassay technique (NEN commercial kit) as method published by Haber *et al.* (1969). No modifications of the commercial kit was introduced. Samples were kept cold and centrifuged within 1 h and the plasma was kept frozen at -20°C until use. All renal values on the same subject were determined in the same incubation and referred to the standard curve, to minimize the error of the method. Plasma renin activity was expressed as $\text{ng}^{-1}\text{h}^{-1}$. Under these conditions the error of the method is $\pm 6.1 \text{ ng}^{-1}\text{h}^{-1}$ when measuring values in the normal range ($0.4\text{--}1.6 \text{ ng}^{-1}\text{h}^{-1}$).

Statistical methods: Descriptive methods were used in the statistical analysis of the data. The difference between circulatory data obtained during different blood volume distribution for each subject analysed with Student's T -test for paired observations. The following probability (P) levels of significance were used:

*** (α), $P < 0.01$ (**), $P < 0.05$ (*). P values > 0.05 are considered insignificant.

Results

Haemodynamic effects of lower body negative pressure (LBNP) -40 mmHg

Seven of the eight subjects LBNP caused a progressive fall in blood pressure, resulting in lightheadedness and necessitating an abrupt termination of the stimulus. These two subjects have been excluded from the statistical analysis.

Five subjects maintained a relative circulatory steady state during LBNP; thus only minor differences are found between the values obtained during the 5th to 9th min compared with the 15th to 19th min of LBNP. The mean of the measurements during the two periods is presented (Table 1). LBNP induced a significant increase in heart rate and a significant increase in pulse pressure, while mean arterial pressure was essentially unchanged. Central venous pressure, stroke volume and cardiac output decreased significantly. Total peripheral vascular resistance increased significantly. Forearm blood flow decreased and regional vascular resistance increased significantly (Fig. 2).

Haemodynamic effects of lower body positive pressure (LBPP) $+40 \text{ mmHg}$

LBPP induced a significant but transient increase in central venous pressure. Thus, during LBPP a significant elevation of central venous pressure compared with the control period

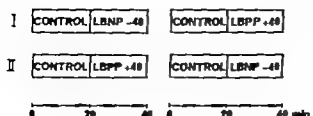


Fig. 1. General experimental design. Cases 3, 5 and 7 were examined according to scheme I, cases 2, 4, 6 and 8 according to scheme II. The heavy lines indicate the time when hemodynamic data are collected.

any detectable change in arterial blood pressure, pulse pressure, dp/dt or HR (Zoller 1972, Johnson *et al.* 1974) suggesting that the vasoconstriction is mediated via receptors on the low pressure side of the circulation.

A temporary increase of venous return and CVP elicited by passive lifting of the leg results in a dilatation of the resistance vessels of the forearm that does not correlate with the magnitude of the increase in arterial pressure and HR, suggesting that the vasodilatation is mediated via low pressure baroreceptors (Roddie, Shepherd and Whelan 1957).

Data thus support the hypothesis that intrathoracic receptors exert important regulatory effects on the sympathetic tone of resistance vessels. Recent studies suggest that such receptors also mediate regulatory effects of the release of renin from the kidney (for review see Davies and Freeman 1976).

The aim of the present investigation was to study central and peripheral circulation during changes in the distribution of blood volume, with the intention of influencing mainly receptors in the low pressure system.

Material

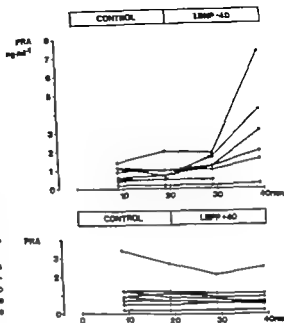
Eight healthy male blood donors participated in the study. In keeping with institutional policy the application was submitted to and approved by the Ethical Committee of Södersjukhuset. The donors informed about the nature and purpose of the investigation. Mean values \pm S.D. of age, weight and height were 31.6 ± 8.8 years, 73.0 ± 7.7 kg and 1.79 ± 0.08 m.

Procedure

The left brachial artery was catheterized percutaneously using a soft teflon catheter (i.d. 1.40 mm). The central venous pressure was obtained by percutaneous introduction of a small teflon catheter into an antecubital vein. The position of the catheter tip was checked by recording respiratory changes typical for intrathoracic recordings.

The lower body (below the iliac crest) was enclosed in an air tight box. With the aid of a high capacity electric pump, the pressure in the box could be changed rapidly to 40 mmHg above or below atmospheric pressure. This procedure will be referred to as lower body negative pressure (LBNP -40) and lower body positive pressure (LBPP +40) respectively. The temperature in the box could be maintained essentially constant during LBNP -40 and during periods with atmospheric pressure in the box. During prolonged LBPP +40, however, measurement in 2 subjects revealed a slight gradual increase in body temperature due to efflux from the pump (approximately 4-5°) during the stimulation period. The measurements were performed with the subjects lying supine in a room with a temperature of about 22°C.

The experimental design is presented in Fig. 1. Every subject was examined during four periods, lasting 20 min and differing only with respect to the pressure surrounding the lower body. The sequence of LBNP -40 and LBPP +40 was alternated in every second case according to Fig. 1. Measurements presented in this study were collected in the period 5-9 min and 15-19 min. In the 10th and 19th



Effect on PRA of LBNP and LBPP. The dotted lines refer to the two subjects with impending syncope. The open circles in the picture below refer to subjects who were investigated according to 11 in Fig. 1. In these subjects the values may be influenced by the long LBNP procedure.

the two subjects with impending syncope, the plasma renin activity pattern during the studied was similar to that in the six subjects who maintained a relative circulatory stable (Fig. 3).

The increase in plasma renin activity during the LBNP period showed a positive correlation to the initial plasma renin activity value ($r = 0.92$) during the preceding control period (4).

Discussion

Lower body negative pressure (LBNP) results in sequestration of some of the blood volume in the lower part of the body. This pooling arises from an increase in the transmural pressure in the lower extremities, mainly resulting in an increase of the amount of blood pooled in the veins. The amount of blood pooled during LBNP -40 has been estimated to be in the order

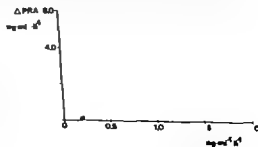


Fig. 4 Increase in PRA during LBNP as a function of the initial PRA value. The regression is calculated as the difference between the value obtained at 15-19 min of LBNP and the mean of the two values during control conditions.

TABLE I Effect of LBNP -40 (n=6) and LBPP +40 (n=8) on some hemodynamic variables. Mean \pm S.E.

	Control	LBNP -40	Diff	Sign. of diff	Control	LBPP +40	Diff	Sign. of diff
Mean arterial pressure (mmHg)	93.5 ± 2.5	93.8 ± 2.9	+0.3 ± 0.1	n.s.	94.6 ± 2.3	99.0 ± 2.6	+4.4 ± 1.2	p < 0.05
Pulse pressure (mmHg)	49.8 ± 1.9	43.0 ± 3.0	+6.8 ± 1.7	p < 0.05	50.5 ± 2.1	51.4 ± 3.2	+0.9 ± 2.5	n.s.
Central venous pressure (mmHg)	6.5 ± 1.0	2.1 ± 1.3	-4.3 ± 0.5	p < 0.01	7.5 ± 0.7	8.8 ± 1.0	+1.3 ± 0.6	n.s.
Cardiac output (l min ⁻¹)	6.4 ± 0.3	5.1 ± 0.2	-1.2 ± 0.3	p < 0.01	5.9 ± 0.4	6.2 ± 0.5	+0.3 ± 0.3	n.s.
Heart rate (beats min ⁻¹)	71.8 ± 1.7	83.7 ± 3.1	+11.8 ± 1.8	p < 0.01	69.3 ± 2.6	70.3 ± 2.1	+1.0 ± 1.6	n.s.
Stroke volume (ml)	88 ± 5	63 ± 4	-26 ± 1	p < 0.01	86 ± 7	89 ± 8	+3 ± 3	n.s.
Total peripheral vascular resistance (units)	13.7 ± 0.6	17.8 ± 0.7	+4.1 ± 0.7	p < 0.01	15.2 ± 1.2	15.9 ± 1.1	+0.7 ± 0.7	n.s.

was found only in the period 5-9 min after the onset of LBPP (+2.1 mmHg, p < 0.05). In the period 15-19 min, central venous pressure was essentially restored to control rate. Comparing the mean values during LBPP with those during the preceding control period (Table I) showed a slight but significant increase in mean arterial pressure (+4.4 mmHg, p < 0.05). No significant change was found with respect to heart rate, pulse pressure, stroke volume, cardiac output or total peripheral vascular resistance. Forearm blood flow increased significantly resulting in a significant decrease in regional vascular resistance (Fig. 2).

Effect of lower body positive pressure (LBPP) and lower body negative pressure (LBNP) on plasma renin activity

LBPP induced no significant changes in plasma renin activity (Fig. 3). After 9 min LBNP there was only a slight increase in plasma renin activity whereas after 19 min plasma renin activity was increased to approximately three times the control value.

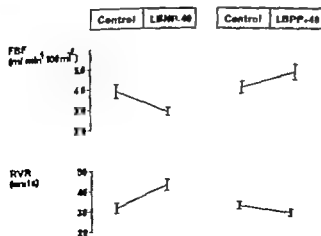
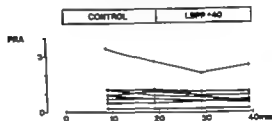
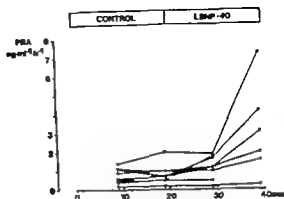


Fig. 2. Effects of LBNP (n=6) and LBPP (n=8) on peripheral circulation. Values \pm S.E. are given. Asterisk denotes significance of deviation. Forearm blood flow (FBF). Regional vascular resistance (RVR).



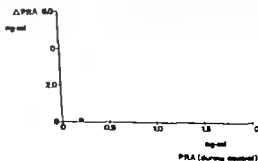
Effect on PRA of LBNP and LBPP
The dotted lines refer to the two subjects developing syncope. The open circles in the picture below refer to subjects investigated according to I in Fig. 1. In these subjects the values may be influenced by the LBNP procedure.

In two subjects with impending syncope, the plasma renin activity pattern during the study was similar to that in the six subjects who maintained a relative circulatory state (Fig. 3).

The increase in plasma renin activity during the LBNP period showed a positive correlation with the initial plasma renin activity value ($r = 0.92$) during the preceding control period (1).

Discussion

Body negative pressure (LBNP) results in sequestration of some of the blood volume in the lower part of the body. This pooling arises from an increase in the transmural pressure in the affected vessels, mainly resulting in an increase of the amount of blood pooled in the lower extremities. The amount of blood pooled during LBNP -40 has been estimated to be in the order



4. Increase in PRA during LBNP as a function of the initial PRA value. The values are calculated as the difference between the values obtained at 15-19 min of LBNP and the mean of the two values during the control conditions.

of 0.5–0.6 l (Murray *et al.* 1967, Musgrave *et al.* 1969). LBNP –40 should therefore be an adequate stimulus for studying the effects of a moderate central hypovolemia in intact man.

The demonstrated reduction in central venous pressure by approximately 4 mmHg elicited by LBNP –40 and the reduction in cardiac output by 1.2 l min⁻¹ are in accordance with earlier studies (for ref. see Wolthuis, Bergman and Nicogossian 1974). Mean arterial pressure was essentially unchanged, indicating a substantial active constriction of the resistance vessels, reflected in the significant increase in both total peripheral and regional vascular resistance.

No direct measurements on the redistribution of blood elicited by lower body positive pressure (+40 mmHg) during steady state conditions have been found in the literature. In a study of the central hemodynamic effect in supine position of inflating an airtight suit covering the legs and the lower abdomen to 80 mmHg, Gray *et al.* (1969) demonstrated only a transient increase in central blood volume, reflected in a short lasting elevation of central venous pressure.

In the present study central venous pressure after an initial rise, showed a similar gradual decrease towards control value. However 5–9 min after the onset of LBPP there was an elevation of 2.1 mmHg ($p < 0.05$), suggesting that the present method of applying lower body pressure around the whole lower body may be slightly more effective than the airtight suit.

LBNP and LBPP elicited opposite changes, i.e. a decrease and an increase respectively in forearm blood flow. Although a slight increase in mean arterial blood pressure was found during LBPP in this material, the unchanged mean arterial pressure during LBNP suggests that the recorded changes in forearm blood flow cannot simply be attributed to changes in mean arterial pressure, i.e. perfusion pressure, but also depend on reflexogenic changes in constrictor tone.

Even if the changes in mean arterial pressure are small, it is probable that changes in arterial baroreceptor activity secondary to, for example, changes in heart rate, pulse pressure, dp/dt or intrathoracic blood volume, may contribute to the demonstrated changes in regional vascular resistance. However a direct stimulation of the carotid sinus by neck suction (Bevegård *et al.* to be published) or manual compression of the carotid artery (Rodde *et al.* 1957), simulating a considerable increase and decrease respectively in intrasinus pressure, results in only small changes in regional resistance in a forearm segment. This suggests that the demonstrated changes in the forearm blood flow are only partially due to changes in the carotid sinus baroreceptor activity.

Recent studies indicate that the vasoconstriction in the forearm during LBNP is mediated mainly via intrathoracic receptors sensitive to changes in central venous pressure and in central blood volume (Zoller *et al.* 1972, Johnson *et al.* 1974). It has also been suggested that low pressure baroreceptors are more important than the carotid baroreceptor for the increase in forearm vascular resistance during up-right tilt (Abboud *et al.* 1975). Murray *et al.* (1967) showed that LBNP caused only minor and inconsistent changes in intrathoracic pressure as assessed from intracosophageal pressure, whereas central venous pressure showed a consistent decrease. This indicates that LBNP decreases the transmural pressure of the right atrium and central veins which may be the stimulus that elicits the vasoconstriction in resistance vessels.

ve leg elevation to drain blood into the thorax, a stimulus that is probably akin to the LBPP used in this study. Roddie *et al.* (1957) implicated that low pressure receptors mediate a reflex vasodilatation in the forearm when the intrathoracic pressure increases. The present finding of an increase in forearm blood flow and a regional vascular resistance might partially be explained by such a mechanism. Sequence of changes during prolonged LBPP stimulus suggests, however, that the forearm blood flow during LBPP in this study cannot depend exclusively on this mechanism. If the vasodilatation depended solely on low pressure baroreceptor-mediated decrease in central venous pressure during the continuous LBPP should be paralleled by gradual decrease in forearm blood flow whereas a slight increase was found. The explanation might be an increase in the skin blood flow due to itching (Crossley *et al.* 1966, Detry *et al.* 1972), as the temperature around the lower limb slightly during the stimulation period. The change in forearm blood flow during LBPP probably reflects the net result of interacting thermo- and baroreceptors. Increase in plasma renin activity during LBPP agrees with the findings by Fasola and (1972) and Mancía, Romero and Shepherd (1976). The release of renin from the kidney is influenced by complex sympathetic and intrarenal mechanisms (Davis *et al.* 1976). Increase in plasma renin activity during LBPP in this study might be elicited by changes in sympathetic stimulation of the juxtaglomerular apparatus and/or changes in renal artery constriction influencing intrarenal mechanisms.

Stimulation of circulatory receptors affecting renin release is controversial. Some suggest that the carotid baroreceptor (Hodge, Lowe and Vane 1966, Bunting, Page and Ross 1966) may influence renin release, but reports in the literature are contradictory—Brennan *et al.* (1974) recently demonstrated that pressure changes in the carotid sinus do not have any demonstrable effect on plasma renin activity in dogs. Brennan *et al.* have demonstrated that receptors on the venous side of the circulation, possibly in the striatum, are involved in the regulation of renin release and Mancía *et al.* (1975) showed that vagal afferents from cardiopulmonary receptors influence the release of renin. It seems probable that the LBPP stimulus used in the present study affects receptors on the venous side distinctly more than those on the high pressure side, as mean arterial pressure was unchanged. This supports the hypothesis that the release of renin is elicited by low pressure receptors.

A significant increase in plasma renin activity was demonstrated only at the end of the 15 min period (Fig. 3), indicating that the rapid increase in total peripheral vascular resistance and in regional resistance was not related to the vasoconstrictive effect of angiotensin II, which is known to be a potent vasoconstrictor. Also, the finding of large inter-individual variations in the increase in plasma renin activity during LBPP (Fig. 3), not related to the changes in total peripheral vascular resistance or regional vascular resistance, suggests that angiotensin II contributes little to the increased vascular resistance during LBPP. This is in accordance with the finding of Kala, Fyhrquist and Ekalo (1974), suggesting that angiotensin II does not play a significant role in the maintenance of blood pressure immediately after the change of posture from supine to upright. The two subjects with a history of fainting syncope showed much the same plasma renin activity response as the subjects

with a relative stable circulation, further supporting the hypothesis that an angiotensin mediated vasoconstriction is of little importance for short-term adjustment of peripheral vascular resistance.

Angiotensin II is also a stimulus to an increased aldosterone secretion and in this way participates in the regulation of the blood volume. A nervous mechanism mediated through the sympathetic renal nerve influences plasma renin activity during blood volume changes. Also intrarenal mechanisms may contribute (Castenfors, Johanson and Orö 1973). The determinant of the large individual variation in plasma renin activity during LBNP in this study was the initial plasma renin activity value under basal conditions (Fig. 4). Sodium balance and intravascular volume are important for the regulation of the renin response to acute postural changes (Tuck, Dluhy and Williams 1975). All subjects were on free salt and water intake and thus interindividual variations in sodium balance may contribute to the great variations in plasma renin activity response to LBNP.

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Effects of Carbachol and Calcium on the Cyclic Guanosine-3',5'-Monophosphate (Cyclic GMP) Metabolism in Intestinal Smooth Muscle

By

KARIN B. NILSSON and ROLF G. G. ANDERSSON

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Abstract

NILSSON K B and R. G. G. ANDERSSON *Effects of carbachol and calcium on the cyclic guanosine-3',5'-monophosphate (cyclic GMP) metabolism in intestinal smooth muscle*
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In a submaximal concentration carbachol contracted the rabbit colon muscle and increased the cyclic GMP level. The cyclic AMP level was reduced. In a Ca^{++} -depleted muscle carbachol reduced the cyclic GMP level while the effect on the cyclic AMP content of the muscle was unchanged. Carbachol had no effect on the guanylate cyclase activity of the "plasma membrane fraction" (the 35-45% fraction) in the homogenate and the microsomal fractions Ca^{++} had no effect on the guanylate activity while it stimulated the enzyme in a soluble fraction. In the "plasma membrane fraction" cyclic GMP released Ca^{++} from the preloaded fraction and inhibited the Ca^{++} accumulation. These effects were not found in the vesicular microsomal fraction (the 35% fraction). In both fractions, however, cyclic GMP counteracted the stimulating effect of cyclic AMP. These results indicate that cyclic AMP and cyclic GMP may have antagonistic roles on the Ca^{++} metabolism in the colon muscle. It is suggested that cyclic GMP may act as some kind of positive feedback mechanism which may have a modulating effect on the release of Ca^{++} from one pool to another in rabbit colon.

There is accumulating evidence that the relaxing action induced by β -adrenoceptor agonists is mediated by cyclic AMP (Andersson 1972, Bär 1974). Contracting drugs, on the other hand, have variable effects on the cyclic AMP levels in different smooth muscles (Andersson 1972, Namm and Leader 1976). In some smooth muscle preparations, however, the contraction seems to be associated with an increase in the cyclic GMP concentration (Goldberger *et al* 1975). Thus, it was early found that cholinergic agonists increased the cyclic GMP level in guinea pig ileum (Lee *et al* 1972). Subsequently Schultz *et al* (1973) found that several contracting drugs increased the cyclic GMP level in smooth muscles and they found this effect to be Ca^{++} -dependent.

To elucidate the role of cyclic GMP in rabbit colon muscle, we have studied the effect of carbachol and calcium on the guanylate cyclase activity in this preparation. This work also deals with the effect of cyclic GMP on the calcium metabolism in a microsomal fraction from rabbit colon.

Methods

Small smooth muscle was prepared from rabbit colon. The rabbit was stunned by a blow to the neck, bled, and the colon muscle was dissected free of tissues and pieces of the muscle were attached directly to plastic holders and immersed in Krebs-Henseleit bicarbonate buffer at $+37^\circ\text{C}$ as described earlier and Mölne-Lundholm (1966). The tension was recorded by FTO3 transducers on Grass graph. In some experiments the muscle was incubated in Ca^{++} -free Krebs-Henseleit bicarbonate buffer. In some experiments the muscle preparations were treated several times with 2×10^{-4} M EDTA to reduce the Ca^{++} in the muscle before the addition of the drug. At different times after the addition of carbachol to muscle they were frozen in Freon 12 and solid CO_2 . The colon muscles were homogenized in 5% dioxane and the cyclic nucleotides were separated by chromatography on columns of AG-1 X8 (4 × 40 cm) ion exchange resin and lyophilized. The cyclic GMP content was estimated according to Saloner *et al.* (1970) and the cyclic AMP according to Gilman (1970). The guanylate cyclase activity was determined by incubation buffer containing 40 mM Tris-HCl (pH 7.4), 2 mM GTP, 10 mM theophylline, 0.1 mM MgCl_2 and other additions as indicated in the figure legends. The reaction was initiated by the addition of 100 μg Tris-extracted proteins from different colon muscle fractions (see below). After incubation at 37°C for 5 min, the reaction was terminated by the addition of 5% PCA. The formed cyclic GMP was separated and analyzed as described above.

Ca^{++} accumulated microsomal subfractions were isolated according to Carsten (1969) with some changes (Månson *et al.* 1976). The crude microsomal fraction isolated between 17 300–40 000 g suspended in 0.05 M NaCl and 0.005 M $\text{Na}_2\text{-oxalate}$ (pH 7.0), and placed on a discontinuous sucrose gradient containing 35%, 45% and 55% sucrose. The gradient was centrifuged for 120 min at 30 000 g . Protein layers were obtained at 35%, 45–48% and the 45–55% sucrose respectively. In this study we used the fractions isolated at 35% and 45–48% sucrose.

The supernatant from the 40 000 g centrifugation was centrifuged at 100 000 g for 60 min and the pellet was used in the guanylate cyclase assay. The homogenate and the soluble fraction used in assay were dispersed in Triton X 100 to final Triton concentration of 1% and kept at $+4^\circ\text{C}$ for later use.

Ca^{++} accumulation studies were performed in an incubation medium containing 0.11 M KCl, 0.01 M CaCl_2 , 0.02 M histidine buffer (pH 7.2), 0.35 mM MgCl_2 , 0.35 mM ATP and 9×10^{-4} M Ca^{++} (adjusted using absorption spectroscopy) and containing 0.008 μCi ^{45}Ca . The tests were performed at $+37^\circ\text{C}$, initiated by the addition of 100–150 μg protein to the medium and interrupted after fixed time intervals by filtration through Millipore filter (0.45 μm). The accumulation of Ca^{++} to the microsomes was determined by liquid scintillation technique and was calculated from the activity in the unfiltered and the filtered fractions. Correction was made for Ca bound to the filter. The protein content was estimated according to Lowry *et al.* (1951).

Statistical analysis. Mean values are given \pm standard error of the mean. The control and the treated values in every separate experiment were obtained from the same animal or microsomal preparation. The significance of the effect was calculated by Student's *t*-test from the difference between these paired samples.

Results

Effect of carbachol on the cyclic nucleotide metabolism in the intact colon muscle

Carbachol in a concentration of 4.5×10^{-4} M elicited a submaximal contraction of rabbit colon muscle. The contraction was associated with a 15% reduction ($p < 0.05$) of the muscle level of cyclic AMP after 10 s confirming earlier results on this preparation (Anderson 1973). This effect was still present after 60 and 120 s. The muscle content of cyclic GMP was increased by carbachol treatment, an increase of about 50% ($p < 0.05$) from a basal value of 42.5 ± 6.8 pmol/g wet weight was found 10 s after the addition of the drug, an effect which was also found after 60 and 120 s (Fig. 1). In calcium-depleted muscle preparations the carbachol induced effect on the cyclic GMP content was reversed (Fig. 1) while the effect on cyclic AMP was still present.

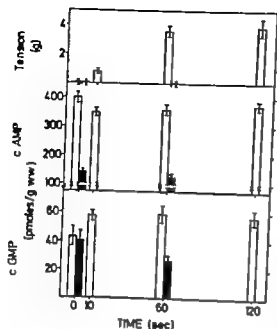


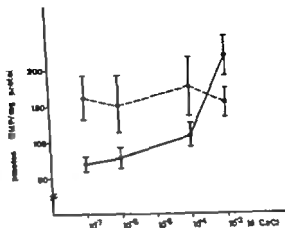
Fig. 1 Influence of carbachol ($4.5 \cdot 10^{-5}$ M) on tension, the cyclic AMP and the cyclic GMP content of normal (open bars) and Ca^{2+} -poor (filled bars) rabbit colon muscle. Mean \pm S.E. (n = 7).

Effects of carbachol and calcium on the cyclic GMP formation of subcellular fractions of rabbit colon

From rabbit intestinal smooth muscle two submicrosomal fractions, the 35% and the 35–45% fraction had been isolated. The biochemical and the morphological investigations indicated that the 35% fraction was of vesicular nature and originated from the plasma membrane as well as the sarcoplasmic reticulum. The 35–45% fraction was a nonvesicular membrane fraction probably mainly consisting of fragments of the plasma membrane (Andersson and Nilsson 1976).

In a previous study (Andersson *et al.* 1975) we have found that carbachol reduced the concentration of cyclic AMP in the colon as well as the activity of adenylate cyclase in the 35–45% microsomal fraction. Since carbachol increased the cyclic GMP content of the colon muscle it was of interest to investigate its effect on the guanylate cyclase activity. In the 35–45% microsomal fraction 4.8 ± 1.0 pmol cyclic GMP was formed per mg protein/5 min. Carbachol did not significantly change the guanylate cyclase in this fraction and the activity was 4.3 ± 0.5 pmol/mg protein/5 min in the presence of the cholinergic agonist.

Since carbachol caused an increase of the cyclic GMP level which was found to be Ca^{2+} dependent (Fig. 1), we have therefore studied the effect of Ca^{2+} on the guanylate cyclase activity in different subfractions from colon. As indicated in Fig. 2 Ca^{2+} ions in the concentration range $5 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ M had no effect on the guanylate cyclase activity of a Triton-dispersed homogenate. The guanylate cyclase activity differed in the two microsomal fractions: most of the activity was found in the 35% fraction, 26.3 ± 5.1 pmol/mg protein/5 min, while the activity of the 35–45% fraction was only about a quarter of that of the 35% fraction (Table I). Calcium in the concentration range 10^{-5} – 10^{-4} M was examined and was found to have no significant effect on the guanylate cyclase activity of the microsomal fractions (Table I).



2 Effect of CaCl_2 ($5 \cdot 10^{-7}$ – $5 \cdot 10^{-2}$ M) on the formation of cyclic GMP in postmitochondrial homogenate (—) and stable fraction (---). Mean \pm S.E. (3–7).

Most of the guanylate cyclase activity of the colon homogenate was found in the 100 000 g postmitochondrial fraction. Lower concentrations of Ca^{++} ($5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ M) did not increase the cyclic GMP formation of the fraction (Fig. 2). Higher concentrations of Ca^{++} (10^{-4} and $5 \cdot 10^{-4}$ M) significantly increased the guanylate cyclase activity. In comparison to activity in the absence of added Ca^{++} was estimated to be 68.3 ± 6.9 pmol/mg protein.

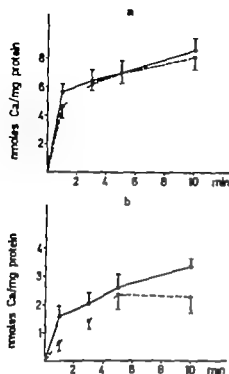
The effect of cyclic GMP on the accumulation and the release of Ca^{++} in the microsomal fractions

The accumulation of Ca^{++} to the microsomal fractions of 35% and 35–45% sucrose was stimulated by cyclic AMP (10^{-6} M) (Nihman 1973). It was noticed that the addition of cyclic GMP to the corresponding fractions had a tendency to reduce the Ca^{++} -binding capacity (Fig. 3). In the 35% sucrose fraction cyclic GMP (10^{-6} M) initially reduced the Ca^{++} -binding but did not influence the maximal binding capacity of the fraction (Fig. 3 a). Cyclic GMP treatment of the 35–45% fraction tended to reduce the Ca^{++} -binding within the space of 1 minute and this tendency was observed even after 10 min of incubation with the nucleotide ($p < 0.1$) (Fig. 3 b).

As the cyclic GMP level of the intact muscle was increased during the contraction it was of interest to investigate whether cyclic GMP influenced any calcium-releasing mechanism

TABLE I. The effect of different concentrations of Ca^{++} on the cyclic GMP formation in microsomal fractions isolated from rabbit colon. The incubation time was 5 minutes. Mean \pm S.E. — 6–7

Experimental conditions	Cyclic GMP formation (pmol/mg protein, 5 min)	
	35	35–45
Blank values	26.3 ± 3.1	4.0 ± 1.5
CaCl_2 ($1 \cdot 10^{-6}$ M)	31.5 ± 6.2	5.0 ± 0.9
CaCl_2 ($1 \cdot 10^{-4}$ M)	27.0 ± 4.7	6.3 ± 1.4
CaCl_2 ($1 \cdot 10^{-2}$ M)	25.6 ± 3.1	3.7 ± 1.0



3

Fig. 3. The effect of 10^{-8} M cyclic GMP (---) on the Ca^{++} binding (a) the 35% fraction and (b) the 35-45% fraction. The control curve is denoted by —. Mean \pm S.E. ($n=6-7$).

Fig. 4. The effect of 10^{-8} M cyclic GMP (---) on the Ca^{++} release from (a) the 35% fraction and (b) the 35-45% fraction. The fractions were preloaded with ^{45}Ca for 5 min before the addition of cyclic GMP. Mean \pm S.E. ($n=6-9$). The effect of cyclic GMP on the Ca^{++} release was significantly separated from the control curve (—) (Fig. 4 b). * $p < 0.05$, ** $p < 0.01$.

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in the colon. The addition of cyclic GMP to the microsomes preloaded by Ca^{++} for 5 min caused a release of Ca^{++} from the 35-45% sucrose fraction (Fig. 4 b). Initially the release was very rapid and was probably significant already after 1 min treatment compared to the control expts. A further release was observed after 3 to 10 min incubation. In the 35% sucrose fraction cyclic GMP (10^{-8} M) had no effect on the Ca^{++} release (Fig. 4 a).

An antagonistic effect of cyclic GMP on the cyclic AMP stimulated Ca^{++} accumulation

George *et al* (1973) reported that cyclic GMP added simultaneously with cyclic AMP reduced the Ca^{++} -binding capacity of the cardiac microsomes compared with cyclic AMP alone. This data support the hypothesis that cyclic AMP and cyclic GMP may play opposite roles in the regulation of cardiac contraction. It was of interest to investigate whether there existed a similar relationship in intestinal smooth muscle microsomes. Simultaneous addition of cyclic GMP ($1 \cdot 10^{-7}$ M to $1 \cdot 10^{-8}$ M) and cyclic AMP ($1 \cdot 10^{-7}$ M) to colon microsomes was performed and the effects were compared with Ca^{++} -accumulation in the presence of cyclic AMP alone. Already at the concentration ratio of 100:1 of cyclic AMP:cyclic GMP a moderate antagonistic effect was observed in the 35% sucrose fraction (Table II). The reduction of the Ca^{++} -accumulation was about 20-30%.

† The influence of cyclic GMP on the Ca^{++} accumulating microsomal fractions of rabbit colon muscle incubated in the presence of cyclic AMP. The fractions having been incubated for 5 minutes in the presence of these agents. Mean \pm S.E. - \pm . Significance degree - $p < 0.05$

used conditions		Reduction of the Ca^{++} accumulation (%)	
AMP	Cyclic GMP	II	35-45
(M)			
10^{-4}		19 ± 3	30 ± 17
$5 \cdot 10^{-4}$		12 ± 4	30 ± 13
$1 \cdot 10^{-3}$		19 ± 6	30 ± 18
$5 \cdot 10^{-3}$		22 ± 9	30 ± 18
$1 \cdot 10^{-2}$		32 ± 11	35 ± 19

the fraction 35-45% increase the stimulating effect of cyclic AMP was significantly the ratio of 20:1 (Table II). The inhibition was optimal at the ratio 10:1 and estimated to be about 30%.

Discussion

enhancement of the cyclic GMP level of the intact colon muscle during contraction by acetylcholine was Ca^{++} -dependent. This is in accordance with the findings on rat duodensum (Schultz *et al.* 1973), where several agents that increased the cyclic GMP level have a Ca^{++} requirement for their action. Cholinergic agonists increased the cyclic GMP level in smooth muscles too. Lee *et al.* (1972) have observed this effect in guinea pig ileum and (1974) reported a similar effect from rabbit gallbladder. Angles d'Auriac and Worcel (1975) did not find any increase in the rat uterus after carbachol administration, however there are thus some observations indicating an elevation of the cyclic GMP level in smooth muscles from the gastrointestinal tract after stimulation with cholinergic drugs.

Our study does not support a hormone-receptor mediated activation of particulate guanylate cyclase, since no effect of carbachol was observed on the guanylate cyclase of the microsomes. The present results and those of Schultz *et al.* (1973) indicate that the most likely soluble cyclase activating factor is Ca^{++} . The soluble guanylate cyclase from colon muscle is stimulated by high concentrations of Ca^{++} -ions ($5 \cdot 10^{-4}$ M, Fig. 2). Calcium, in relatively high concentrations, increased the soluble guanylate cyclase activity of sea urchin sperm (Garbers *et al.* 1975) as well as of rat brain (Olsson *et al.* 1976). The concentrations of Ca^{++} that are required for demonstrating effects on guanylate cyclase are so high that its physiological importance becomes unclear. It must be concluded, however, that the incubation conditions used in most *in vitro* studies are not identical with the "milieu intérieur" of smooth muscle cells, and the enzymatic activities *in vitro* may therefore have different requirements of calcium.

The physiological role of cyclic GMP in smooth muscle contraction is unclear. We have suggested that cyclic GMP may promote contraction by acting as a modulator on Ca^{++} release in the smooth muscle cell (Andersson *et al.* 1975). Contradictorily cyclic GMP has been suggested as a negative feed-back signal which tends to lower the cytosolic Ca^{++} .

concentration (Schultz *et al* 1975). The results of the present investigation provide a theoretical basis for cyclic GMP as a modulating factor for Ca^{++} release in colon muscle since the nucleotide caused a release of Ca^{++} from one of the microsomal subfractions (Fig. 8). Cyclic GMP also counteracted the stimulating effect of cyclic AMP on the Ca^{++} accumulation in colon microsomes (Table II) which is in conformity with the observations on enteric microsomes (George *et al* 1973).

The results presented earlier by Schultz *et al* (1973) and Andersson *et al* (1976) along with the findings in this work have led us to the working hypothesis. The interaction of cholinergic agonists with muscarinic receptors on the colon muscle membrane causes a depolarization and an increase of the myoplasmic Ca^{++} concentration. Ca^{++} ions may stimulate soluble guanylate cyclase and increase the cyclic GMP level. The elevation of cyclic GMP may be a factor of importance for the maintenance of contraction by supporting the Ca^{++} release from certain subcellular calcium-pools.

It appears likely that cyclic GMP may be a modulating factor in rabbit colon contraction. We have to be cautious in our generalizations, since the action of cholinergic agonists are without effect on cyclic GMP levels of uterine smooth muscle Angles d'Auriac and Worcel (1975). Further in indomethacin-treated guinea-pig gallbladder we have found that the contraction induced by cholecystokinin is unaffected although the increase of cyclic GMP has failed to appear (Andersson *et al* 1976).

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Antilipolytic Effect of Adenosine in Dog Adipose Tissue *in Situ*

By

BERTIL B. FREDHOLM and ALF SOLLEVI

Adenosine and related compounds are released from isolated rat fat cells (see Schmal *et al* 1975) and from canine subcutaneous adipose tissue (Fredholm 1976). The rate release from the latter preparation is enhanced by sympathetic nerve stimulation. Adenosine inhibits cyclic AMP accumulation in isolated rat fat cells already in submicromolar concentrations (Eain *et al* 1972, Schwabe *et al* 1975), while lipolysis is inhibited to a considerable lower degree in this preparation. As discussed by Fain (1973) this apparent contradiction might be explained if cyclic AMP levels are raised to supramaximal levels by the concentration of the lipolytic drugs commonly used in studies of isolated fat cells. In a recent study we found that maximal rates of lipolysis in canine subcutaneous adipose tissue induced by nerve stimulation are associated with only small (50%) increases in tissue cyclic AMP concentration (Fredholm *et al* 1976).

In the present study we have therefore investigated the effect of adenosine on lipolysis induced by sympathetic nerve stimulation. A preliminary account of some of these findings has been given (Fredholm 1975).

21 fed, female mongrel dogs weighing 8-16 kg were used. Anesthesia was induced with 30 mg/kg and pentobarbital and supplemented as necessary during the experiment. Subcutaneous adipose tissue was isolated (Rosell 1966) and autoperfused. Responses were studied in 30 fat pads weighing 20-42 g. The pads were trephotomized, artificially ventilated and arterial pO_2 , pCO_2 and pH controlled. Heparin (2000 U/kg) was given to prevent clotting. Adipose tissue blood flow was measured using a drop counter. The preparation was isolated and stimulated at 4 Hz (2 ms, 12 V). Arterial and venous blood samples were drawn and glycerol (Laurell and Tibblin 1966) was assayed on deproteinized plasma. Adenosine was administered by close i.a. infusion. For further experimental details see below. Dipyrindol (Persantin, Boehringer Ingelheim) was administered i.v.

Adenosine infused i.a. to give concentrations in the arterial blood flow ranging between 0.01 and 40 μM had no significant effect on the basal rate of glycerol release. In contrast, glycerol release evoked by electrical nerve stimulation was inhibited by adenosine in concentrations above 1 μM (Table I).

In order to test the possible significance of endogenous adenosine the well-known adenosine uptake inhibitor dipyrindol (Schrader *et al* 1972) was administered to the dogs. The results of these experiments are summarized in Table II. While ineffective on basal glycerol release, dipyrindol (0.5 mg/kg i.v.) inhibited stimulated glycerol release. The present results demonstrate that exogenous adenosine is antilipolytic in canine subcutaneous adipose tissue *in situ*. A 70% inhibition was found by administering adenosine

TABLE I Inhibition of stimulated glycerol release by adenosine. $\bar{x} \pm S.E.$

Adenosine concentration (μM)	Glycerol release (% of control)
0	100 \pm 6
10	101 \pm 6
20	117 \pm 14
40	74 (61, 87)
80	27 \pm 10 ($p < 0.001$)

Isolated glycerol release was calculated by summing the total amount of glycerol released during and during nerve stimulation and subtracting the basal release for the same overall time period.

TABLE II Effect of dipyridamol (Persantin®) on glycerol release from canine subcutaneous adipose tissue $\bar{x} \pm S.E.$

	Before dipyridamol	After dipyridamol ^a	Per cent of control
Basal release rate (nmol min^{-1} 100 g^{-1})	0.153 \pm 0.026 (11)	0.156 \pm 0.026 (7)	100 \pm 16
Stimulated release (nmol 100 g^{-1})	21.6 \pm 3.4 (11)	13.3 \pm 3.6 (7)	57.4 \pm 10.6 (7)
		$p = 0.01$	$p < 0.01^b$

^aDipyridamol was administered i.v. in a dose of 0.5–1 mg/kg before nerve stimulation.

^bSignifies t-test for means of two samples.

^cSignifies t-test for one sample.

rate calculated to give 10–40 μM concentration in the blood. Since adenosine is rapidly inactivated in blood and tissue (Schrader *et al.* 1977) the concentration of adenosine achieved in the fat cells is probably considerably lower. In isolated rat fat cells noradrenaline stimulated lipolysis was inhibited by less than 25 per cent at 10 μM adenosine (Hjemdahl and Fredholm 1976). Thus adenosine is a far better inhibitor of lipolysis *in vivo* than *in vitro*. Since adenosine probably acts by inhibiting cyclic AMP formation (Fain 1973; Schwabe *et al.* 1975; Hjemdahl and Fredholm 1976) this may be related to the fact that cyclic AMP increases *in vivo* are far smaller than those commonly studied *in vitro* (Fredholm *et al.* 1977).

The recent finding that adenosine inhibits noradrenaline release from several tissues including canine subcutaneous adipose tissue (Hedqvist and Fredholm 1976) could also actually explain the potent antilipolytic effect of adenosine under the present experimental conditions.

Adenosine (and related compounds) is found in the venous effluent following nerve stimulation (Fredholm 1976). It can be calculated that the maximal concentrations achieved are in the μM range (Fredholm *et al.* 1977). Since the present results demonstrate that adenosine is antilipolytic under these conditions, a physiological role must be considered. Therefore the finding that dipyridamol, a well-known inhibitor of adenosine inactivation (Schrader *et al.* 1977), decreased the lipolytic response to nerve stimulation might be interpreted as evidence for a role of endogenous adenosine as a feed back inhibitor of lipolysis. Further proof of this hypothesis is necessary, however.

This study was supported by the Swedish Medical Research Council (proj. no 64X 2553) and by Karolinska Institute.

Antilipolytic Effect of Adenosine in Dog Adipose Tissue *in Situ*

By

BERTIL B. FREDHOLM and ALF SOLLEVI

Adenosine and related compounds are released from isolated rat fat cells (see Schriber *et al* 1975) and from canine subcutaneous adipose tissue (Fredholm 1976). The rate of release from the latter preparation is enhanced by sympathetic nerve stimulation. Adenosine inhibits cyclic AMP accumulation in isolated rat fat cells already in submicromolar concentrations (Fain *et al* 1972, Schwabo *et al* 1975) while lipolysis is inhibited to a considerable lower degree in this preparation. As discussed by Fain (1973) this apparent contradiction might be explained if cyclic AMP levels are raised to supramaximal levels by the combination of the lipolytic drugs commonly used in studies of isolated fat cells. In a recent study we found that maximal rates of lipolysis in canine subcutaneous adipose tissue induced by electrical stimulation are associated with only small (30%) increases in tissue cyclic AMP concentration (Fredholm *et al* 1976).

In the present study we have therefore investigated the effect of adenosine on lipolysis induced by sympathetic nerve stimulation. A preliminary account of some of these results has been given (Fredholm 1975).

21 fed, female mongrel dogs weighing 8-16 kg were used. Anaesthesia was induced with 30 mg/kg pentobarbital i.v. and supplemented as necessary during the experiment. Subcutaneous adipose tissue was isolated and a segment was superfused. Responses were studied in 30 fat pads weighing 70-42 g. The dogs were tracheotomized, artificially ventilated and arterial pO_2 , pCO_2 and pH controlled. Heparin (200 U/kg) was given to prevent clotting. Adipose tissue blood flow was measured using a drop counter. The preparation was isolated and stimulated at 4 Hz (2 ms, 12 V). Arterial and venous blood samples were drawn and glycerol (Laurell and Tibbling 1966) was assayed on deproteinized plasma. Adenosine was administered by close i.a. infusion. For further experimental details see below. Dipyrindamol (Pernianin Boehringer Ingelheim) was administered i.v.

Adenosine infused i.a. to give concentrations in the arterial blood flow ranging between 0.01 and 40 μM had no significant effect on the basal rate of glycerol release. In contrast, glycerol release evoked by electrical nerve stimulation was inhibited by adenosine in concentrations above 1 μM (Table I).

In order to test the possible significance of endogenous adenosine the well-known adenosine uptake inhibitor dipyrindamol (Schrader *et al* 1972) was administered to the dogs. Results of these experiments are summarized in Table II. While ineffective on basal glycerol release, dipyrindamol (0.5 mg/kg i.v.) inhibited stimulated glycerol release. The present results demonstrate that exogenous adenosine is antilipolytic in canine subcutaneous adipose tissue *in situ*. A 70% inhibition was found by administering adenosine

TABLE I Inhibition of stimulated glycerol release by adenosine. $\bar{x} \pm S.E.$

Adenosine concentration (μ M)	Glycerol release (% of control)
1 (4)	100 \pm 6
6-8.15 (6)	101 \pm 6
10 (8)	117 \pm 14
20 (10)	74 (41, 87)
40 (8)	27 \pm 10 (p < 0.001)

Isolated glycerol release is calculated by summing the total amount of glycerol released during and during nerve stimulation and subtracting the basal release for the same overall time period.

TABLE II Effect of dipyrindamol (Permantol[®]) on glycerol release from canine subcutaneous adipose tissue ($\bar{x} \pm S.E.$)

	Before dipyrindamol	After dipyrindamol ^a	Per cent of control
ad adipose tissue			
rest 100μ M	0.153 \pm 0.026	0.156 \pm 0.026	100 \pm 16
	(11)	(7)	
stimulated adipose	21.6 \pm 3.4	13.3 \pm 3.6	57.4 \pm 10.6
rest 100μ M	(11)	(7)	(7)
		p < 0.01	p < 0.01 ^b

Dipyrindamol was administered in doses of 0.5-1 mg/kg before nerve stimulation.

Student's t-test for means of two samples

Wilcoxon t-test for one sample

rest calculated in give 10-40 μ M concentration in the blood. Since adenosine is rapidly activated in blood and tissue (Schrader *et al.* 1972) the concentration of adenosine achieved in the fat cells is probably considerably lower. In isolated rat fat cells noradrenaline stimulated lipolysis was inhibited by less than 25 per cent at 10 μ M adenosine (Hjendahl and Fredholm 1976). Thus adenosine is a far better inhibitor of lipolysis *in vivo* than *in vitro*. Since adenosine probably acts by inhibiting cyclic AMP formation (Fain 1973, Schrambe *et al.* 1975, Hjendahl and Fredholm 1976) this may be related to the fact that cyclic AMP increases *in vivo* are far smaller than those commonly studied *in vitro* (Fredholm *et al.* 1977).

The recent finding that adenosine inhibits noradrenaline release from several tissues including canine subcutaneous adipose tissue (Hedqvist and Fredholm 1976) could also partially explain the potent antilipolytic effect of adenosine under the present experimental conditions.

Adenosine (and related compounds) is found in the venous effluent following nerve stimulation (Fredholm 1976). It can be calculated that the maximal concentrations achieved are in the μ molar range (Fredholm *et al.* 1977). Since the present results demonstrate that adenosine is antilipolytic under these conditions, a physiological role must be considered. Therefore the finding that dipyrindamol, a well-known inhibitor of adenosine inactivation (Schrader *et al.* 1977), decreased the lipolytic response to nerve stimulation might be interpreted as evidence for a role of endogenous adenosine as a feed back inhibitor of lipolysis. Further proof of this hypothesis is necessary, however.

The study was supported by the Swedish Medical Research Council (proj. no. 04X 2557) and by Karolinska Institute.

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The Effect of Oxygen on Peripheral Airways

By

TAGE MORS NIELSEN and OLE FRED PEDERSEN

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Abstract

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In normal young subjects four alveolar O_2 -concentration levels ranging from about 7 to 95 were attained by breathing different concentrations of oxygen in nitrogen at constant alveolar CO_2 -concentrations of about 5%. Maximum expiratory flows at 40% TLC were measured at each level and corrected for the influence of the expired gas density on flow. The corrected maximum flows are not significantly different, which is taken as evidence of no effect of oxygen on tone of peripheral bronchi.

Key words: Bronchomotor effect of O_2 , O_2 tension, maximum expiratory flow-volume curves

It has previously been shown that hypocapnia causes a progressive decrease of maximal expiratory flow at midlung volume, indicating constriction of peripheral bronchi (Nielsen and Pedersen 1976 b). This effect is of significance in maintaining a normal ventilation/perfusion ratio, as it may help to decrease wasted ventilation of poorly perfused areas in the lungs. Similarly the pulmonary vasoconstriction caused by hypoxia helps to decrease wasted perfusion of poorly ventilated areas (*cf.* review by Hughes 1975).

Whether oxygen also is of significance in maintaining normal ventilation in relation to perfusion is not known, even though its influence on bronchomotor tone has been investigated earlier. A review of the literature has been given by Barer *et al.* (1972) and by Widdicombe (1963). In normal subjects there seems to be no influence of hyperoxia on airway resistance (Boiler *et al.* 1960, Astin and Pennan 1967). Hypoxia, however, seems to cause a fall in specific airway conductance both in normal subjects (Sterling 1964) and in patients with chronic bronchitis (Astin and Pennan 1967).

These measurements of resistance and conductance are mostly influenced by conditions in larger airways, however, and do not give special information about the influence of O_2 on smaller airways, where the effector sites must obviously be, if oxygen should be effective in changing local ventilation in relation to perfusion. The effect here of hyperoxia should then be bronchoconstriction and the effect of hypoxia bronchodilatation, which is apparently not the case for the total airway (*cf.* above).

In the previous study we showed that hypocapnia caused a progressive decrease of maximal expiratory flow in 60% total lung capacity (V_{\max} (60% TLC)). The purpose of the present investigation has been to examine the effect on V_{\max} of varying alveolar oxygen tensions (P_AO_2) under conditions with unchanged alveolar carbon dioxide tensions (P_ACO_2). By using the same subjects as previously and, so far as possible, the same experimental procedure, it should furthermore be possible to compare the effect, if any, of changing P_AO_2 with the effect of changing P_ACO_2 , both within physiological ranges.

Material and methods

Ten young healthy subjects (two females and eight males) were investigated. None of them had a history of heart or lung disease. The subjects were the same as presented in an earlier paper (Nielsen and Pile 1976 b).

The experimental set-up and the procedure were with a few exceptions identical with those used in previous investigation and have previously been described in detail. Therefore only a brief account is given here.

In a randomised way four series of maximum effort flow-volume (MEFV) curves were made for each subject each series with different alveolar oxygen concentration (F_AO_2) produced by letting the subject respire gas mixtures with 12.5, 20.95, 29.25 and 100% O_2 in N_2 in series I, II, III, and IV respectively. In each series five blows were made.

As in the previous study end-tidal CO_2 concentrations (F_ACO_2) were measured continuously they were displayed for the subjects, and these were asked to ventilate to $F_ACO_2 = 5\%$. In the present study average of F_AO_2 of the last 10 expirations before the blow was furthermore measured by taking out 10 ml of gas, 10 ml at a time, from the expiratory tube during the inspiration. This gas was passed through an oxygen analyzer (Servomex, OA 150), which was calibrated with known gases and shown to give response linearly related to the O_2 percentage.

On the MEFV curves, displayed on a storage oscilloscope and photographed with polaroid can V_{\max} (60% TLC) was measured, converted to BTPS, and corrected for the influence of density using formulas described previously (Nielsen and Pedersen 1976 a).

The corrected V_{\max} (from hereon called V_{\max} only) from the 4 series and the 10 subjects were compared by a two-way analysis of variance (anova) with replication (Sokal and Rohlf 1969), using the same procedures and significance levels as in the previous study.

Results

In Table I are presented mean V_{\max} and standard deviations for each series and subject. It is seen that the individual differences between the series are small and that the difference between the grand mean for each series is indeed very small. This is confirmed by the analysis of variance which shows that there is no significant difference between the four series, the ratio of mean squares (F_0) being 1.29 with 3 and 27 degrees of freedom (df). The difference between subjects is highly significant however F being 1169.39 with df = 9 and 160.

It was possible to avoid an influence of CO_2 on V_{\max} . The grand means of F_ACO_2 for subjects in series I, II, III and IV respectively were 4.82%, 4.89%, 4.85%, and 4.81%. These values were not significantly different from each other as F was 0.78 with df = 3 and 27.

The mean values of F_AO_2 in series I, II, III and IV respectively were 7.24%, 15.17%, 23.87%, and 95.09%.

1. The mean of the corrected \dot{V}_{max} (cf text) for each subject in the 4 series. In brackets are shown the standard deviations. For comparison is shown the grand mean of uncorrected V_{max} in addition.

	Series I ($F_iO_2 = 12.51\%$) V_{max} (l·min ⁻¹)	Series II ($F_iO_2 = 20.95\%$) V_{max} (l·min ⁻¹)	Series III ($F_iO_2 = 29.25\%$) V_{max} (l·min ⁻¹)	Series IV ($F_iO_2 = 100\%$) V_{max} (l·min ⁻¹)
	3.13 (0.07)	3.36 (0.13)	3.25 (0.13)	3.06 (0.14)
	4.62 (0.30)	4.65 (0.07)	4.40 (0.13)	4.38 (0.13)
	3.32 (0.25)	3.15 (0.20)	3.16 (0.26)	3.29 (0.09)
	6.06 (0.22)	5.89 (0.21)	5.83 (0.34)	5.92 (0.30)
	7.24 (0.08)	7.47 (0.18)	7.39 (0.18)	7.49 (0.14)
	6.52 (0.18)	6.64 (0.26)	6.69 (0.29)	6.37 (0.17)
	4.58 (0.23)	4.76 (0.35)	4.61 (0.37)	4.73 (0.13)
	3.89 (0.30)	3.91 (0.24)	4.02 (0.21)	3.73 (0.07)
	2.85 (0.13)	2.84 (0.15)	2.79 (0.13)	2.70 (0.09)
	3.71 (0.25)	3.58 (0.17)	3.61 (0.15)	3.68 (0.07)
nd mean	4.59	4.63	4.58	4.54
nd mean of corrected \dot{V}_{max}	4.65	4.65	4.58	4.33

Discussion

Results thus indicate that changing F_iO_2 from about 7 to 95% in normocapnic conditions does not have any influence on V_{max} (60% TLC), when correction is made for the density of different density of the inhaled gases.

In Table 1 the grand mean of uncorrected V_{max} for each series is shown. The difference between the highest and lowest values is about 7% compared to a difference of about 2% of the grand means of corrected V_{max} (Table 1). As it could be shown by anova that the difference between series of the uncorrected V_{max} was highly significant, it is of importance to use the correction when changing F_iO_2 to avoid a mistake that would have supported the hypothesis that F_iO_2 is of significance in maintaining ventilation in relation to perfusion (cf above).

Compared with the effect of hypocapnia (reducing normal P_aCO_2 by a factor 2 caused decrease of V_{max} by 14%) the influence of O_2 on bronchial muscular tone can only be small. This does, however, not completely exclude a small effect, which would have been revealed, had the method been more sensitive.

Our findings agree with those of Butler *et al* (1960) who found no effect of hyperoxia on airway resistance in healthy subjects whether the effect was of short duration or of 5 to 6 h duration.

Astin and Pennan (1967) as well found no effect on airway resistance, corrected for the influence of thoracic gas volume, of inhalation of 30% O_2 in normal subjects, but they found 20% decrease of resistance in patients with chronic bronchitis. This decrease was correlated to the hypoxemia of the patients before the administration of O_2 . Astin (1970) confirms that the decrease in resistance after 30% O_2 in such patients is related to the hypoxemia before the administration, and not to arterial carbon dioxide tension or arterial pH. Thus chronic hypoxemia in such patients causes bronchoconstriction.

In the previous study we showed that hypocapnia caused a progressive decrease of the maximal expiratory flow at 60% total lung capacity (V_{\max} (60% TLC)). The purpose of the present investigation has been to examine the effect on V_{\max} of varying alveolar oxygen tensions (P_AO_2) under conditions with unchanged alveolar carbon dioxide tensions (P_ACO_2). By using the same subjects as previously and, so far as possible, the same experimental procedure, it should furthermore be possible to compare the effect, if any of changing P_AO_2 with the effect of changing P_ACO_2 , both within physiological ranges.

Material and methods

Ten young healthy subjects (two females and eight males) were investigated. None of them had a history of heart or lung disease. The subjects were the same as presented in an earlier paper (Nielsen and Pedersen 1976 b).

The experimental set up and the procedure were with a few exceptions identical with those used in previous investigation and have previously been described in detail. Therefore only a brief account will be given here.

In a randomised way four series of maximum effort flow-volume (MEFV) curves were made for each subject each series with different alveolar oxygen concentration (F_AO_2) produced by letting the subject respire gas mixtures with 12.5, 20.95, 29.25, and 100% O_2 in N_2 in series I, II, III and IV respectively. In each series five blows were made.

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On the MEFV curves, displayed on a storage oscilloscope and photographed with a polaroid camera \dot{V}_{\max} (60% TLC) was measured, converted to BTPS, and corrected for the influence of density using formulas described previously (Nielsen and Pedersen 1976 a).

The corrected \dot{V}_{\max} (from hereon called V_{\max} only) from the 4 series and the 10 subjects were compared by a two-way analysis of variance (ANOVA) with replication (Sokal and Rohlf 1969), using the same procedures and significance levels as in the previous study.

Results

In Table I are presented mean \dot{V}_{\max} and standard deviations for each series and subject. It is seen that the individual differences between the series are small and that the difference between the grand mean for each series is indeed very small. This is confirmed by the ANOVA which shows that there is no significant difference between the four series, the ratio of mean squares (F_0) being 1.29 with 3 and 27 degrees of freedom (df). The difference between subjects is highly significant, however F being 1169.39 with df = 9 and 160.

It was possible to avoid an influence of CO_2 on V_{\max} . The grand means of F_ACO_2 for subjects in series I, II, III and IV respectively were 4.82%, 4.89%, 4.85%, and 4.8%. These values were not significantly different from each other as F_0 was 0.78 with df = 3 and 27.

The mean values of F_AO_2 in series I, II, III and IV respectively were 7.24%, 15.11%, 23.87%, and 95.09%.

Renal Hemodynamics in the Perinatal Period

A Study in Lambs

By

ANITA APERIA, OVE BROMERIÖ, PETER HEILIN and INGEMAR JOELSSON

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Abstract

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Perinatal changes in renal hemodynamics have been studied in lambs. Eleven of the lambs were exteriorized, but maintained on placental circulation (fetal lambs). Eight of the lambs were delivered with cesarean section and studied immediately after clamping of the cord (new-born lambs). Nine lambs were delivered naturally and studied during the first 9 days of life. Renal blood flow (RBF) was determined by the streptokinase method using samples drawn from the aortic artery as the reference flow. The filtering capacity of the nephrons was evaluated after mercurysulphate injection and dissection of the nephrons. Total RBF did not seem to change much at birth but increased in relation to kidney weight during the first postnatal week. Clamping of the cord did, however, result in a change in intrarenal blood flow distribution, so that in new-born lambs relatively more of the blood flow was perfusing the outer cortical region. During the first postnatal week there was a slight, but significant further relative increase in outer cortical blood flow. The relative increase in outer cortical blood flow at birth was accompanied by an increased frequency of having superficial nephrons from 22 to 77 %. Practically all juxtamedullary nephrons were filtering before birth. Three days postnatally 93 % of the superficial and 100 % of the juxtamedullary nephrons were filtering.

The fetal kidneys are known to produce urine already during the second third of gestation in those mammals where intramammary recordings have been performed (Alexander and Nixon 1961). The degree of renal maturation reached at birth appears to be inversely related to the homeostatic efficiency of the placenta. In all species, however, the placenta is the chief homeostatic organ during intrauterine life.

The aim of the present study has been to describe by which means the kidney will meet the requirements of extrauterine life. Special regard has been paid to renal hemodynamics since renal blood flow is of utmost importance for the postnatal renal functional development (Aperia *et al.* 1974). Total renal blood flow, intrarenal blood flow distribution and the filtering capacity of the nephrons have been determined in lamb fetuses just before term, in newborn lambs immediately after the umbilical cord was clamped and in 1-9 days old lambs.

Material and Methods

General preparation. The experiments were performed in twenty-eight lambs. Nineteen of those lambs were delivered with cesarean sections and studied as fetuses or new-borns. In eight lambs the cord was

In normal subjects, Sterling (1968) found 22% fall in specific conductance of the π after inhalation of 10–12% O_2 .

Compared to these results ours indicate that there is no influence of neither hypoxia or hyperoxia on tone in peripheral airways in normocapnic normal young subjects. This is necessarily in contrast to those of Sterling, but rather provides the new information that change in conductance found by him is due to a change of tone in larger bronchi and not of tone in smaller bronchi.

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I General hemodynamic measurements. Oxygen tension (PO_2) and mean arterial blood pressure.

	Fetus	Newborn	1-3 days	6-9 days	31-48 days
mmHg	23.7 \pm 3.30	42.0 \pm 8.85	N.M.	N.M.	N.M.
pressure, mmHg	64.1 \pm 5.84	73.1 \pm 5.94	86.3 \pm 9.46	98.3 \pm 10.8	107.1 \pm 5.67

N.M., not measured.

perical and juxtaglomerular nephrons were those defined by Bovalet *et al.* (1972). The superficial π cells would occupy the outer 14% and the juxtaglomerular glomeruli the inner 15% of cortical volume. Four superficial and 29 juxtaglomerular nephrons from ten fetal lambs, 35 superficial and 13 juxtaglomerular nephrons from 6 newborn lambs and 18 superficial and 9 juxtaglomerular nephrons from three 1-3 day lambs were inspected. Five of the 34 superficial nephrons in the fetal group were directed into the distal tubule. In most cases camera lucida drawings at the magnification 50-80 \times of the glomerulus and the entire proximal tubule, and the size of the ferricyanide precipitate indicated

vascular counting. Just before sacrificing the animals, 3-10 ml Indasink was given in the aorta just above the renal arteries for color marking the glomeruli of the remaining right kidney. The right kidney was removed and sectioned for determination of intrarenal blood flow distribution and number of units. The kidney was divided transversely in an upper and lower half. One of the halves was only chosen for counting its total include amount for the purpose of estimating total RBF. The other was cut frontally in 2-3 about 1 mm thick sections. Each of these sections as sliced under dissection scope site at outer and inner part, each occupying approximately 10-15%, 60% and 25% of total volume. Radioactivity was measured by gamma spectrometer (Packard scintillation spectrometer) with lead shielded ventilation detector. In 3 fetal, 4 newborn and in 2 and 4 lambs of 1-3 and 6-9 days of age, respectively slices from outer and inner cortex were also used for glomerular counting using a modified method by Demichels *et al.* (1945). The estimation of the density of perfused glomeruli calculation of the average blood flow to single glomeruli units. It was assumed that all spheres trapped in the glomeruli. All renal tissue pieces were wet weighed before radioisotope and glomerular counting.

The renal blood pressure was monitored by Statham transducer and recorded on a Grass model 7B strip. The blood pressure did not decline during the experiment and was not significantly affected by injection of microsphere and ferricyanide. PO_2 was determined in arterial blood samples using Clark oxygen electrode. The values presented in Results were sampled immediately before injection of microsphere. For statistical calculations, Student's *t*-test has been used.

Results

The perinatal changes in arterial oxygen tension and mean arterial blood pressure are illustrated in Table I. Arterial oxygen tension (PO_2) was only recorded in the fetal and newborn lambs. As expected, clamping of the cord resulted in a highly significant ($p < 0.001$) increase in arterial PO_2 . Clamping of the cord also resulted in an increase in arterial blood pressure that was significant ($p < 0.01$). The arterial blood pressure continued to increase almost linearly during the first 10 days of life. Thereafter the blood pressure appears to have reached a steady level, since the mean pressure recorded in 31-48 days old lambs (Aperia and Herin) is 107.1 ± 5.7 mmHg, which is not significantly different from the value recorded in 6-9 days old lambs ($p > 0.5$).

Fig. 1 shows the average changes in kidney weight and glomerular density during the perinatal period. The kidney weight was slightly lower in the fetal lambs than in the newborn lambs. During the first postnatal week there was approximately fifty per cent increase

clamped and the animals were allowed to breathe before the study (newborns). In addition, two days old and five 6-9 days old lambs were studied. The body weights of the fetal and newborns averaged 3 kg, of the 1-3 days old lambs 3.8 kg and of the 6-9 days old lambs 4.2 kg. The gestation age of the animals delivered with cesarian sections ranged between 135-145 days estimated from the for artificial fertilization and maternal physical signs. Normal gestation time in sheep is 145-150. The pregnant ewes were allowed free access to food and water until 6 h before study. The ewes were atropine (1 mg) and were anesthetized with a small dose of Pentothal sodium (6-7 mg/kg). Flunitrazepam (Pavulon, 1.5 mg) was administered for muscle relaxation. The ewes were ventilated known gas mixtures through an endotracheal tube connected to an Engström respirator. Arterial samples were taken repeatedly to control PO_2 within adequate limits (80-120 mmHg) (Jodsson *et al.* 1973). A 3% glucose solution was continuously infused (150 ml/h) during the experiment. Through a left peritoneal incision the uterine wall was opened carefully to avoid placental bleeding. After delivery all lambs were placed on a thermoregulated table in order to maintain the initially recorded rectal temperature ranged between 38.5-39.5°C. This is generally considered to be the optimal temperature of ewe lambs (Joelsson *et al.* 1970). After exteriorizing the fetus different procedures were used in the group the heads of the animals were immediately after exposure covered with a saline filled rubber avoid air breathing. The umbilical cord was kept intact and prevented from stretching and cool the newborn lambs spontaneous respiration was started immediately after the umbilical cord was cut. In local anesthesia polypropylene (pp) catheters were inserted into the femoral arteries (pp 90). In one femoral catheter was, during continuous pressure recordings, introduced into the left where it was permanently placed, or withdrawn to a place just above the aortic valves. The other artery catheter was advanced only a few cm (iliac catheter). The preparation of the lamb described completed within 8 min after delivery.

Lambs studied in the later postnatal period (four 1-3 days and five 6-9 days of age) were kept and artificially ventilated as described above for the ewes. The lambs were catheterized in the iliac in the fetal and newborn groups.

Renal blood flow determinations with microsphere method. Renal hemodynamics were studied by the microsphere method. Spheres with a diameter of $15 \pm 3 \mu m$ and labeled with a radioisotope of ^{51}Cr were suspended in dextran (10%) with Tween 80 added. A homogeneous solution is obtained by ultrasonic treatment (Branson Sonic Power Company). Immediately thereafter 0.6-0.8 ml of the sphere solution or total number of around 700 000 microspheres was injected during about 15 s. Injection site in the left ventricle or just above the aortic valves should provide adequate mixing (Fry *et al.* 1973, Kleinman and Reuter 1973). Microspheres with a diameter of $15 \mu m$ are thought to be distributed within the kidney according to the renal blood flow and will almost exclusively be trapped in the glomerular capillaries during the first renal circulation (Bockberg *et al.* 1971, Kat *et al.* 1971). The number of spheres in renal tissue will therefore be proportional to blood flow in corresponding areas.

The absolute intrarenal blood flow was measured by means of reference blood sample (Andersson 1973). This sample was withdrawn by a constant speed pump (3 ml/min) (Sage Instruments) from the aorta during 25 s from time of microsphere injection. As no blood is added to the aortic blood flow at the entrance of the renal arteries to the sampling site, there should be similar concentrations of spheres in blood perfusing the kidneys as in the reference sample. The ratio of nuclei amount in tissue and blood flow to corresponding tissue area would then be in proportion to the ratio of amount in the reference blood sample and the sampling rate (Le 3 ml/min).

Determination of filtering capacity. The ability of the glomeruli to filter was determined with ferric as an indicator. The left kidney was exposed through flank incision. 0.8 ml of a 10% ferrocyanide solution was rapidly injected into the aorta just above the renal arteries. The transit time from the injection site to the glomerular capillaries had previously been determined (range 1-3 s) by inspecting the renal cortex following the injection of 0.2 ml of 10% solution of indocyanine green. After 15 s after the time of the ferrocyanide injection the renal pedicle was abruptly clamped. The kidney was then rapidly excised and frozen in fluid nitrogen. The ferrocyanide remaining in the kidney was precipitated by Prussian blue in ferric chloride solution at -11°C during 18 h. This procedure was followed by washing in 20% HCl at 37°C for 475 h. The macerated kidney was kept in preservation solution (70% in 95 ml ethanol and 5 ml conc. HCl) at +4°C until dissected. Complete superficial and junctional proximal tubules including pars recta were dissected under stereomicroscope, placed in a drop of water and inspected for the presence of Prussian blue particles, i.e. the ferrocyanide precipitate. In order to determine the filtering capacity the ferrocyanide should be entirely recovered in the tubules. Only those tubules were used where the ferrocyanide precipitate was intensely blue and could easily be observed. The

B. Absolute values (mean \pm S.D.) of renal hemodynamics.

	Renal blood flow ml/g renal tissue /min	Glomerular blood flow ml/min outer cortex	mid cortex	inner cortex
(a) 5)				
mm	1.35	23.2	21.8	31.6
)	± 0.25	± 6.46	± 2.95	± 6.43
mm (a=4)				
mm	1.61	34.5	22.3	28.5
)	± 0.44	± 10.85	± 5.80	± 6.86
mm (a=4)				
mm	1.75	78.8	63.8	39.8
)	± 0.47	± 22.4	± 5.91	± 8.66
mm (a=5)				
mm	1.91	93.2	61.6	30.0
)	± 0.43	± 23.8	± 18.4	± 13.0

in newborn group. Postnatally however RBF/g will increase from birth to 6-9 days (a). This increase in RBF/g is quite remarkable, since at the same time there is also a decrease in kidney weight.

The blood flow to the superficial glomeruli increased at birth both in absolute terms (Table II) and in relation to the blood flow to the juxtamedullary glomeruli (Fig. 3). The ratio between inner and outer cortical (IC/OC) tissue flow was obtained in all twenty-eight lambs studied. The IC/OC ratios of GBF and tissue flow were significantly lower in the newborn than in the fetal lambs ($p < 0.001$ and $p = 0.01$ respectively). During the first postnatal week there was a slight further fall in the IC/OC ratio of GBF and tissue flow. The ratios obtained in the newborn lambs were, however not significantly different from those recorded in the 6-9 days old lambs ($p > 0.5$). Nor were they significantly ($p > 0.5$) different from those recorded in the 40 days old lambs (Aperia and Herin). In fact, the IC/OC ratio in the 40 days old lambs was slightly higher than the ratio in the 6-9 days old lambs.

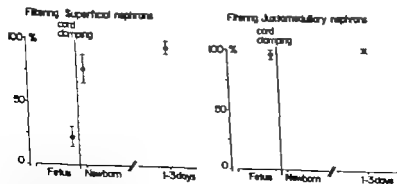


Fig. 3. The relationship between the number of filtering nephrons and the number of superficial nephrons (Fig. 3 a) and juxtamedullary nephrons (Fig. 3 b) studied. The dots represent the mean value in each age group and the bars are standard deviation.

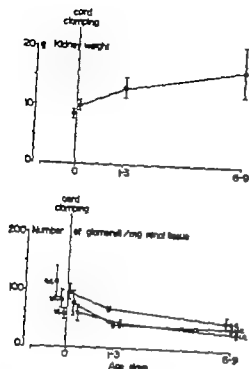


Fig. 1 Right kidney weight and mean number of glomeruli counted per mg renal tissue (glomerular density) in outer (OC), middle (MC), and inner (IC) cortex in fetal, newborn, and in 1-3 and 6-9 days old lambs. The dots represent one standard deviation.

in kidney weight. The increase in kidney weight was accompanied by a fall in glomerular density in all cortical layers. The glomerular density in the 6-9 days old lambs was significantly lower than in the newborn lambs in outer cortex (OC) ($p < 0.001$), middle cortex (MC) ($p < 0.02$) as well as in inner cortex (IC) ($p < 0.01$).

The absolute values for total renal blood flow per gram kidney weight (RBF/g) and glomerular blood flow (GBF) are given in Table II. The results strongly suggest that total RBF will not change much at birth. The RBF/g values were practically identical in the fetal and

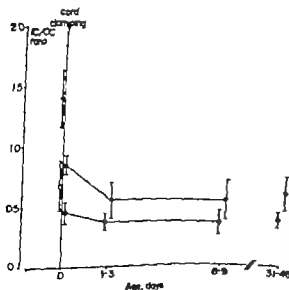


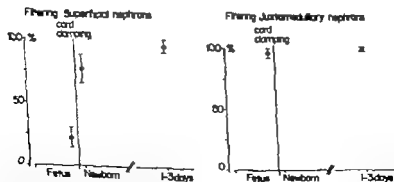
Fig. 2 The relationship between ratio of inner to outer cortical (IC/OC) tissue blood flow (GBF) and glomerular blood flow (GBF) in fetal, newborn and in 1-3, 6-9 and 31-40 days old lambs. The dots represent the mean value in each age group and the bars one standard deviation.

II. Absolute values (mean \pm S.D.) of renal hemodynamics.

	Renal blood flow, ml/g renal tissue/ min	Glomerular blood flow ml/min outer cortex	ml/min inner cortex	inner cortex
(n = 5)				
m	1.55	23.2	21.8	31.6
s	± 0.27	± 6.46	± 2.95	± 6.43
60 (n = 4)				
m	1.61	34.5	22.3	28.5
s	± 0.48	± 10.83	± 5.90	± 6.86
40 (n = 4)				
m	1.75	78.8	63.8	39.8
s	± 0.47	± 23.4	± 9.91	± 8.66
20 (n = 5)				
m	1.91	95.2	61.6	30.0
s	± 0.43	± 23.8	± 18.4	± 13.0

a newborn group. Postnatally however RBF/g will increase from birth to 6-9 days.
 b. This increase in RBF/g is quite remarkable, since at the same time there is also a
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c. blood flow to the superficial glomeruli increased at birth both in absolute terms
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 in 40 days old lambs was slightly higher than the ratio in the 6-9 days old lambs.



2. The relationship between the number of filtering nephrons and the number of superficial nephrons (Fig. 3 a) and juxtamedullary nephrons (Fig. 3 b) studied. The dots represent the mean values in each age group and the bars are standard deviation.

Fig. 3 a and b demonstrate the perinatal changes in the filtering capacity of the nephrons. The nephrons selected were localized with their glomeruli only in the superficial and juxtamedullary cortex. Practically all juxtamedullary nephrons were filtering before birth and remained filtering after birth (Fig. 3 b). The superficial nephrons, however, displayed a high functional heterogeneity prenatally (Fig. 3 a). In each of the ten fetal lambs 3-12 superficial nephrons including complete proximal tubules with attached glomeruli were dissected out. In 4 of the fetal lambs none of the superficial nephrons inspected were filtering, in three of them less than 25% and in the remaining 3 fetuses, 33-70% of the superficial nephrons were filtering. Of the 39 nonfiltering nephrons, 5 were dissected and inspected in the distal tubules to assure that the bolus had not already passed the proximal tubule. In another 11 inspected superficial nephrons (not included in the general calculations) Prussian blue was only located in the glomeruli. In each of the 6 newborn lambs 3-10 superficial nephrons were inspected. In 3 of these lambs, all superficial nephrons were filtering. In the remaining three newborn lambs, 33, 50 and 80% of the superficial nephrons were found to be filtering. In the three 1-3 days old lambs, 16 superficial and 9 juxtamedullary nephrons were dissected and of these, 94 and 100%, respectively, were found to be filtering.

Since the time between the injection of the ferrocyanide and the arrival of the bolus in the glomerular capillaries was most likely not comparable in the different animals, no attempts have been made to make quantitative comparisons of the filtration in the different groups. It might be noted, however, that the mean distance between the ferrocyanide preparation and the glomerulus was in each animal 10-15% larger in the filtering juxtamedullary than in the filtering superficial nephrons.

Discussion

Several methodological problems make interpretation of studies from the perinatal period difficult. The major problems include large individual variations, instability of preparation and large rapid changes in systemic circulation. The large individual variations will in part depend on different conditions in fetal life. The fairly large scatter of the RBF values for both in this and in previous studies on fetal lambs (Beguín *et al.* 1974, Rudolph and Heymann 1967) is therefore not surprising. The renal blood flow values recorded in the fetal lambs were, however, in fairly good agreement with those observed previously (Beguín *et al.* 1974, Rudolph and Heymann 1967).

The mean total RBF value in the fetal lambs did not differ significantly from the total RBF in the newborn lambs. This strongly suggests that total RBF will not change much at birth. During the first week, however, there was a rise in the perfusion rate of renal tissue from 1.61 to 1.91 ml/g/min. In 31-48 days old lambs RBF/g averages 2.54 ml/g/h (Aperia and Herin).

Although cord clamping was not found to change total RBF, it had a pronounced effect on intrarenal blood flow distribution. The quotient between inner and outer cortical glomerular blood flow (IC/OC ratio) decreased significantly at the moment of birth. Such blood flow redistribution has previously been described in puppies but during a considerably longer period of time, *i.e.* during the first 2 weeks of life (Kleinman and Reuter 1973).

the redistribution of intrarenal blood flow seems to be completed in the early postnatal

In 2-79 days old lambs no significant change in inner to outer cortical tissue blood is been observed (Aperia *et al.* 1974). The fact that inner to outer cortical blood flow continues to fall during the first weeks of life in dogs (Jose *et al.* 1971 Klehrman and 1973, Offenberg *et al.* 1973) is most likely due to different stages of renal maturation ages and lambs. The pronounced effect of birth on intrarenal blood flow distribution however well be a general process occurring in all species. The final effect will then depend on the stage of renal maturation at birth. In lambs, the developmental changes of renal blood flow distribution will be almost completed at birth, while in dogs, birth only accelerate the centrifugal functional development.

The rapid change in intrarenal blood flow distribution that occurs when clamping the umbilical cord in part be due to the general circulatory changes that take place at birth (Assali 1962, Barcroft 1947 Dewes 1961). The arterial blood pressure increases significantly at birth (Kilbickog *et al.* 1970). In the adult animal it has been observed by several investigators (Kilbickog 1975, McNay and Abe 1970) that the outer cortical blood flow is more dependent on perfusion pressure than the inner cortical blood flow when the pressure is reduced below autoregulatory range, i.e. about 75 mmHg. In adult rats and dogs a decrease in arterial blood pressure will thus result in a higher inner to outer cortical blood flow ratio. A recent study from this laboratory (Aperia and Herin) in 2-9 days old lambs has shown that a drop in renal artery perfusion pressure to approximately 62 mmHg will cause a reduction in blood flow to the outer and mid cortex only. The inner cortical blood flow will, however, not be significantly changed when the pressure is reduced. During the first postnatal days the arterial blood pressure will continue to increase but there will no longer be any significant effect on intrarenal blood flow distribution. It therefore seems likely that factors other than pressure are also responsible for the redistribution of intrarenal blood flow at birth. The low arterial PO_2 before birth might contribute to the relatively low outer cortical blood flow in the fetal lambs since situations with hypoxia in the adult animals has been observed to result in an increase of perfusion to the inner cortex (Barger 1966). The change in intrarenal hemodynamics occurring at birth might of course also be due to vasoactive substances such as renin. At least the maternal concentration of this substance is high at the time of delivery and it is therefore believed to play an important role in the circulatory adaptation to extrauterine life (Mott 1975).

Clamping of the cord had a dramatic effect on the filtering capacity of the superficial nephrons. Glomerular filtration rate is determined by the product $K_f \cdot P_{G\bar{y}}$. K_f is the product of the hydraulic conductivity and the surface area of the glomerular capillaries. $P_{G\bar{y}}$ is the difference between the hydrostatic (P) and oncotic (π) pressure forces across the glomerular capillary. Since the effect of birth on the glomerular filtration in the superficial nephrons is immediate, it must be due to changes in $P_{G\bar{y}}$ rather than in K_f . The increase in $P_{G\bar{y}}$ is most likely be attributed to the combined effect of an increased hydrostatic pressure in the glomerular capillaries ($P_{G\bar{y}}$) and an increased glomerular plasma flow that will increase the mean oncotic pressure in the filtering glomerular capillaries ($\pi_{G\bar{y}}$). Comparative studies in plasma loaded and hydropenic rats before and after aortic constriction have shown (Robertson *et al.* 1972) that at the lowest recorded values for $P_{G\bar{y}}$ and glomerular plasma

Fig. 3 a and b demonstrate the perinatal changes in the filtering capacity of the nephron. The nephrons selected were localized with their glomeruli only in the superficial and juxtamedullary cortex. Practically all juxtamedullary nephrons were filtering before birth and remained filtering after birth (Fig. 3 b). The superficial nephrons, however display functional heterogeneity prenatally (Fig. 3 a). In each of the ten fetal lambs 3-12 superficial nephrons including complete proximal tubules with attached glomeruli were dissected. In 4 of the fetal lambs none of the superficial nephrons inspected were filtering, in the others, less than 25% and in the remaining 3 fetuses, 33-70% of the superficial nephrons were filtering. Of the 39 nonfiltering nephrons, 5 were dissected and inspected to the distal tubules to assure that the bolus had not already passed the proximal tubule. In most of the inspected superficial nephrons (not included in the general calculations) Prussian blue was only located in the glomeruli. In each of the 6 newborn lambs 3-10 superficial nephrons were inspected. In 3 of these lambs, all superficial nephrons were filtering. In the remaining three newborn lambs, 33, 50 and 80% of the superficial nephrons were found to be filtering. In the three 1-3 days old lambs, 16 superficial and 9 juxtamedullary nephrons were dissected and of these, 94 and 100% respectively were found to be filtering.

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flow P_{oc} will be relatively important for the determination of P_{GFR} . When P_{oc} and plasma flow will increase the importance of the latter factor for the control of GFR increase. This is in accordance with the previous observation from this laboratory, once the filtration is initiated its further development will closely follow that of the glomerular plasma flow (Aperia and Herin 1975).

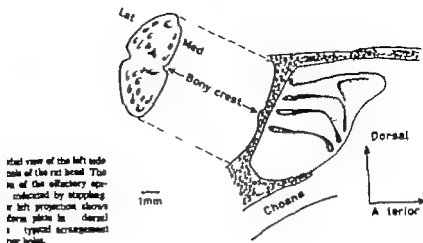
The total RBF did most likely not change at birth. Yet, the intrarenal changes in blood flow and function that occurred might have important consequences for total renal function. The relative increase in the number of filtering superficial glomeruli strongly suggests an increase of the total glomerular filtration from the moment of birth. The increased contribution of the superficial nephrons to total renal function will also influence the work performed by the kidney since there are functional differences between the superficial and the juxtamedullary nephrons both with regard to filtration (Baines 1973, Borvalet *et al* 1972, Jamnik 1970) and tubular function (Borvalet *et al* 1971, Schmidt and Dubach 1971).

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these observations and the observations on the spatial distribution of olfactory bulb axons one can assume a non-homogeneous distribution of receptors within the epithelium. Simultaneous recordings of the electro-olfactogram (EOG) at different sites of the epithelium such a non-homogeneous distribution was demonstrated in frog (Mustajoki, Deval, Levetan and MacLeod 1969).

wanted to compare the pattern of selective degeneration with a possible pattern of homogeneous distribution of receptor cells. In the present study we show the spatial distribution of the different types of receptor cells in the rat by analysis of the relative amplitude of the EOGs recorded from different areas of the cribriform plate.

Materials and methods

Brain of experimental animals was the same as used in the studies on the selective degeneration of the olfactory bulb of rat after prolonged odour exposure (Pantazis and Dawling 1974). White rats of the Wistar strain were used. They were kept in semi-geriatric conditions in isolation units to avoid rhinitis. The rats were between 180 and 230 g (18–23 weeks old) when used in the experiments. Experimental animals were anesthetized with urethane given intraperitoneally (1.5 g per kg b.wt.). The tracheal intubation from the respiration tracheostomy was carried out and polyethylene tube was inserted through the tracheostomy into the choana. The animal was fastened in a headholder. The skull was opened dorsally and the olfactory bulb and the frontal part of the cerebrum was removed. The dorsal surface of the cribriform plate as indicated in Fig. 1. Lymph and cerebro-spinal fluid was continuously removed by cellulose wicks. Deep body temperature of the preparation was monitored at 37.5°C. During unilateral recordings, the contralateral (right) nostril was plugged with vaseline.

Recording

Recording electrodes made contact with the basal side of the olfactory epithelium at the holes of the cribriform plate. Monopolar recordings were made against a common indifferent electrode placed on the skull. The electrodes were broken glass capillaries, tip diameter 25–100 µm, filled with agar Ringer and fixed in electrode holders containing saturated Ag/AgCl (WPI, Harnett, Conn. USA). The potentials were pre-amplified by a DC-amplifier with input impedance of 10 MΩ, and recorded on a pen recorder (KJ Dynagraph 2 channel with DC-coupler, frequency response: DC to 20 Hz).

Spatial Distribution of the EOG in the Rat; a Variation with Odour Quality

By

G THOMMESSEN and K. B. DOVING

Received 1 July 1976

Abstract

THOMMESSEN G and K. B. DOVING *Spatial distribution of the EOG in the rat as a function of odour quality* Acta physiol. scand. 1977 99 270-280.

The spatial distribution of olfactory receptors in the rat has been studied by simultaneous recording electroolfactograms (EOGs) from areas of the olfactory mucosa. The potentials were recorded from the dorsal surface of the cribriform plate, leaving the mucosa and the nasal cavities intact. The ratio between peak amplitude of the EOG potentials served as a measure of the relative sensitivity of one area against another. The results obtained by stimulating with 31 substances at 34 positions demonstrate a non-homogeneous distribution of different receptors. Each substance gives a spatial pattern of response with characteristic peaks. The response distributions are mapped on the cribriform plate for the different odours.

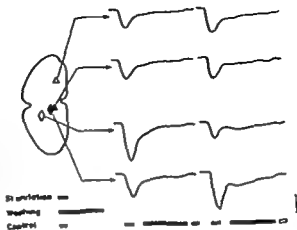
The existence of different olfactory receptors in vertebrates has been demonstrated in (Gesteland *et al* 1963 Altner and Boeckh 1967 Duchamp *et al* 1974) and in the torpid opossum (Mathews 1972). A differential spatial distribution of receptor cells of different specificity to olfactory stimuli is a possible basis for the functional organization of this sensory system within the vertebrates. Adrian (1951) found that the different odours evoked marked responses in different areas of the rabbit olfactory bulb. His findings were confirmed by Mozell and Pfaffman (1954).

The spatial distribution of responses to odours within the olfactory system have also been demonstrated by other methods. Thus prolonged exposure to an odour causes a characteristic pattern of morphological changes named selective degeneration (Doving and Pinching 1973 Pinching and Doving 1974). Odour stimulation is associated with an increased metabolic activity which is unevenly spatially distributed (Sharp *et al* 1975).

Le Gros Clark (1951) made lesions in the glomerulus layer of the olfactory bulb of rabbits. On the basis of the retrograde degeneration patterns in the olfactory mucosa he concluded that the projection of the olfactory epithelium on the olfactory bulb was somatotopic. This was confirmed by Land (1973) looking at the anterograde degeneration in the glomerulus after sectioning of small bundles of the olfactory nerve. The projections showed however to be divergent and partially overlapping.

Limonene methylbenzoate

2. Differences in response to limonene and methylbenzoate in percentages of the mobile electrode as the stationary electrode. Electrode positions are shown to left. Stimulation time is 1 s. rest 1 sec. 0.5 mV



method

From series of control experiments were performed. The stability of the preparation and the experimental conditions were controlled by repeating the first stimulation at the end of each stimulation series. Experiments were made to control the ratio N at various odor concentrations. In 17 series several stimulations were made with the same odor at different intensities. These experiments would indicate possible systematic variations of the ratio N between the amplitudes at the stationary and mobile electrode positions with the concentration of the odor. To control the variability from animal to animal eight pairs of preselected equivalent recording positions were studied.

Results

The extension of the olfactory mucosa on the endoturbinat surfaces was studied in several rats and an average distribution is shown in Fig. 1. The difference between the sensory and the respiratory surfaces is clearly indicated by the ciliary movements in the latter epithelium in a freshly killed animal.

By recording from the dorsal surface of the cribriform plate as described by Ottoson (1954, 1959) the EOGs are recorded without interfering with the air flow in the nasal cavities. EOGs were positive when recorded from the basal side of the olfactory epithelium (Ottoson 1954, 1959) and behaved like a summated generator potential. If the electrode penetrated the mucosa, the EOG became negative.

The time course of the EOG agrees with that described by Ottoson (1954, 1956, 1959). After a latency of approximately 0.2 s the EOG rose to full size in 0.5 to 0.7 s. By prolonged stimulations an adaptation was seen whereby the EOG reduced to about 2/3 of its peak amplitude. Only the peak amplitude has been subject to analysis in the present article. EOG amplitudes to odorous stimulation varied from 0.2 to 2.5 mV. To standardize recording conditions, stimulation procedures were modified so that responses usually did not exceed 1 mV, thereby minimizing stimulation of receptor types with different specificity. Examples of the recordings are shown in Fig. 2.

TABLE 1. List of stimuli.

Order of stimulation	Symbols	Name	Order of stimulation	Symbols	Name
4	C5	Cyclopentanone	17	HOL	1 Heptanol
5	C6	Cyclohexanone	16	ION	β -Iosone
24	C7	Cycloheptanone	11	IBA	Isobornylacetic
6	C8	Cyclooctanone	27 31	IPA	Isopentylacetic
25	C9	Cyclononanone	8	LJM	(+)-Limonene
1	C10	Cyclodecanone	21	MEN	L. Menthol
	C11	Cycloundecanone	13	MBZ	Methylbenzoate
26	C12	Cyclododecanone	22	MBL	2 Methyl-3-butyl-2-ol
3	C13	Cyclotridecanone	27 31	MHN	6-Methyl-5-hepten-2-ol
15	ACE	Acetophenone	23	MUL	2 Methylundecanal
27-31	BEN	Benzylthiol	10	MVA	4-Methyl valeric acid
20	CAM	DL-Camphor	9	NAP	Naphthalene
7	CHL	Cyclohexanol	19	NBZ	Nitrobenzene
18	DEC	1 Decanol	27 31	OCT	Octanal
12	EAA	Ethyl acetosuccinate	14	MCC	1,1,2,2-tetramethyl-3,3-dichloro-cyclopropane
27 31	HAL	Heptanal			

Stimulation

The catheter in the choana was connected to a suction pump via a flowmeter and a magnetic valve which was controlled by a programming device giving 1 s suction, 1 s pause, 5 s suction, $\frac{1}{2}$ s pause in suction. Suction air flow was held at 7-14 ml/s. The preparation was continuously supplied with pure and humidified air through a glass funnel at a rate of 100 ml/s, producing a clean air-smell from snout.

In the studies of selective degeneration (Pinching and Doving 1974) were used 44 different odours. The present investigation 31 of them were found to be potent enough to give basis for discussion. The odours and their order of presentation are listed in Table 1. The stimuli were introduced on polyethylene spatulae between the funnel and the snout just before the first (1 s) suction and withdrawn during first pause. The second ($\frac{1}{2}$ s) suction thus served as washing and the last ($\frac{1}{2}$ s) suction as a control. In every position the total series of stimuli were used. Grading of stimuli was performed by sniffing in position of the spatula in the clean air flow.

Experimental procedure

For positioning the recording electrodes on the preparation the horizontal bony crest divides cribriform plate in two approximately equal parts, served as landmark. One electrode was placed on left cribriform plate in position approximately 0.5 mm ventral to this crest and 1 mm from the left extension as seen in Fig. 2 and 3. This electrode will later be referred to as the 'stationary electrode'. Other recording electrode was successively placed at 38 different positions uniformly spread over the cribriform plate, and will be referred to as the 'mobile electrode'. Every preparation was photographed and the electrode positions were plotted on the photograph. The different electrode positions are shown in Fig. 6a.

Data treatment

The treatment of the results is based on the method used by Mustaparta (1971). The ratio, R , between the EOG amplitude of the mobile electrode and that of the stationary one served as a measure of stimulating efficiency of each substance in the position of the mobile electrode. This procedure was used to minimize the effects of variable concentrations.

The results were transferred to non-parametric scale by ranking the ratios according to decreasing at every recording position. The response in the position of the stationary electrode was quantified as mean rank value for each stimulant. This value is equal to 32 minus the mean of all the rank values obtained for that stimulant and regarded as equivalent to the proper rank numbers. For each odour substance the ranks were noted on position maps similar to those shown in Figs 5 and 6.

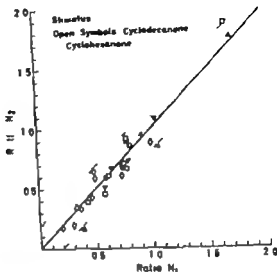


Fig. 4. The ratios N obtained by stimulation with the same substance at the beginning (N_1) and at the end (N_2) of each stimulation series. Open symbols refer to the different preparations as indicated. Fig. 6a. Broken lines show the 95% confidence interval.

N varies from one electrode position to the other. The diagram demonstrates the general tendency of the ratio to be constant over a large range of concentrations for the same stimulus and electrode position.

In 11 runs covering several recording positions, 3–8 consecutive stimulations were made with the same substance. The standard deviation of the ratio N of the EOG amplitude at the mobile electrode to that at the stationary one, with the same substance and position, showed a mean value of 0.07. This standard deviation is less than that obtained for the total series (11 stimuli) in any recording position, which ranged from 0.08 to 0.42, the lowest values in medial positions.

The stability of the preparation and the reproducibility of the results were also controlled by repeating the first stimulation at the end of each series. This was done in all cases except for the 4 ventral positions mentioned above. Fig. 4 shows the ratio N (or the first (N_1) and the last (N_2) stimulation with the same substance in the 34 stimulation series. In all but one case the control stimulus was cyclodecanone. At three positions only the difference between the ratios fell outside a 95% confidence interval found by using the above value of $SD(N)$. At none of the positions included in this presentation the differences fall outside the 98% confidence interval centered at zero. The test confirms that the preparations do not change significantly in sensitivity throughout the experiments.

The results consist of measurements made on five animals, 4 to 12 recording positions in each (Fig. 6a). The positions cover overlapping areas of the cribriform plate. Due to individual variations none of the positions could be regarded as completely congruent in different animals. Some recording positions were however assumed to be equivalent from one preparation to another, indicated by overlapping symbols in Fig. 6a.

We have made eight comparisons, calculating the Spearman rank correlation coefficients. In seven of these comparisons the ranking of odours were positively correlated with signif-

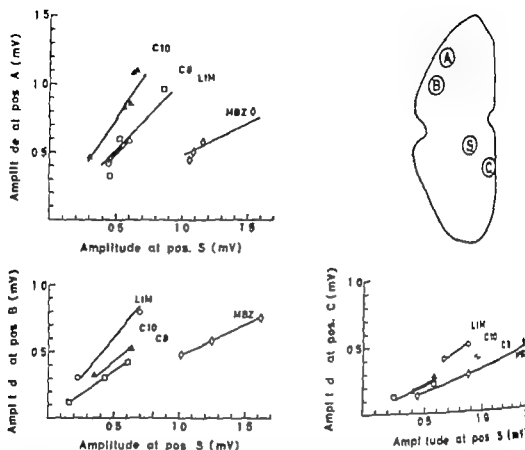


Fig. 3 The peak amplitudes of the electro-olfactogram recorded with different stimulus intensities. recordings at the different positions A, B and C are simultaneous with those at the stationary electrode position, S. Pos. A and pos. B are from the same animal.

The abbreviations in the diagrams refer to the different chemicals listed in Table 1. The straight lines are drawn through the origin. Note the difference in stimulation efficiency of limonene between pos. A and pos. B compared with that of the cycloketones.

At four positions along the ventral margin of the cribriform plate, hatched on the position map Fig. 6a, most responses were too faint to give substantial bases for data treatment.

Purified air elicited responses of 0.1–0.2 mV depending upon the flow of air such presumably due to imperfect purification. This response disappeared only when the actual air flow was insufficient for odour stimulation.

Controls of the reliability of the data

Various expts. were performed to study the reliability of the results. As mentioned in discussion the ratio N between two recording positions should be independent of stimulus intensity. To test this hypothesis repetitive stimulations were made with varying stimulus concentrations. Fig. 3 shows the amplitudes of the EOG at the stationary electrode position versus those from 3 other areas. Plots have been made for four odours, viz. two cycloketones, limonene and methyl benzoate. The ratio N is shown as the slope of the line through the origin. The variation is small with different concentrations but the slopes of the lines

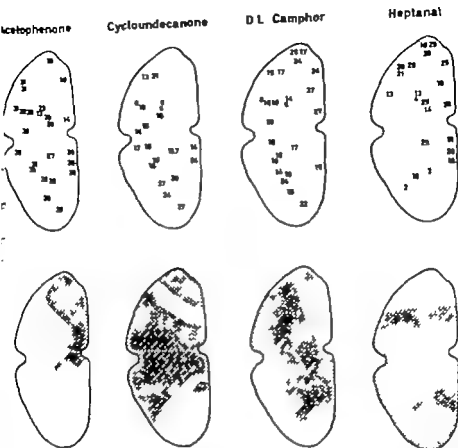


Fig. 5 Examples of response distributions to four different odors. Upper row: the rank numbers obtained at every position. Low rank numbers show high response rates. Lower row: Simplified response distribution maps. Areas with rank numbers lower than 20 are hatched, areas with rank numbers lower than 10 are crosshatched.

response distribution of methylheptanone shows any similarity to that of camphor. The maps obtained for benzylthiole, cyclononanone, ethyl acetacetate, isobornylacetate, isopentylacetate, limonene, methylundecanal, octanal and aceta-methyl-dichloro-cyclo-propane bear the closest resemblance to the heptanal map. The responses to β -ionone, methyl-betynol and heptanol were so uniformly distributed that no pattern will be proposed.

The majority of 26 substances can also be seen to divide in two groups with response distribution maps approximately complementary to each other. One group of 16 odors represented by acetophenone and cycloundecanone, gives the strongest response dorsally or dorso-medially. The group of 10 here represented by heptanal give the strongest responses ventrally or laterally.

Another way of representing the results is shown in Fig. 6. In a map of the cribriform plate the recording positions are marked with different symbols for each preparation (Fig.

incance levels less than 0.5 per cent. In only one comparison concerning positions centrally in the dorsal half of the cribriform plate there was no significant correlation. These results indicate that the results included in this presentation were reproducible from one preparation to another.

Stimulating with different substances

At all the different positions studied the ratio of the EOG amplitude at the mobile relative to the stationary electrode position varied with odour quality. Recordings of the EOG from different positions on the cribriform plate are shown in Fig. 2. Recording pairs with positions marked with the same symbols were recorded simultaneously. As seen limonene gave the smallest amplitude at the stationary electrode position in both cases. Methyl-benzoate gave the highest response at the "mobile" position in the upper pair but for the EOGs at the lower pair of positions, the highest response was at the stationary electrode. Relative differences in stimulation efficiency are also demonstrated in Fig. 4 for limonene and two of the cycloketones.

Comparisons of EOGs from left and right sides

3 series of simultaneous recordings have been made with the 2 recording electrodes symmetrically placed on the left and the right side cribriform plate. In this case, the ratio R was found to be similar for all odours. The variations were not greater than expected from random variations in stimulus concentrations on the two sides, inaccuracy in response measurements or the uncertainty in finding exactly equivalent positions on nearly symmetrical structures. There is therefore no basis for assuming qualitative differences in sensitivity distribution between the olfactory epithelium of the right and the left side.

Mapping of response distribution

The N values of each position of the mobile electrode were ranked, giving the number 1 to the highest ratio and rank number 31 to the lowest. The rank numbers were plotted on the map of the cribriform plate, one map for each stimulus used. 4 examples of such maps are given in the upper row of diagrams in Fig. 5. In an attempt to visualize the sensitivity distribution in the olfactory epithelium as it appears by recording from the cribriform plate, the maps were simplified by hatching. Areas with rank numbers 1-9 have been crosshatched as a sign of high stimulus efficiency. Areas with rank numbers 10-19 have been covered with simple hatching and areas with rank numbers 20-31 are unhatched as signs of medium and low response respectively. In Fig. 5 this arbitrary and simplified visualization of the data is shown for acetophenone, cycloundecanone, camphor and heptanal.

The four maps represent 4 different types of distribution pattern which are fairly typical. Of the remaining 27 substances, 24 gave distribution maps which had visual similarity to one of the four maps shown. Similarity to the acetophenone map was found for the lower cycloketones of 5-8 carbon atoms, cyclohexanol, menthol, methylbenzoate, 4-methyl valeric acid, naphthalene and nitrobenzene. Responses to decanol, cyclodecanone, cyclododecanone and cyclotridecanone were distributed similarly to the map for cycloundecanone. Only the

in variation in the electrical noise of the recording devices and the uncertainty in the location of the potentials.

EOG may be recorded anywhere in the proximity of the olfactory epithelium. The most convenient recording site in the mammal is the nearly two-dimensional cribriform plate. In the described preparation procedure the EOG potentials are recorded without interference with the nasal flow pattern since the nasal cavities are intact. The recordings are also free of electrode potential artifacts as the electrode tips are not exposed to the stimulating odors.

The recorded potentials are electrotonic projections of potentials from a highly folded olfactory epithelium serving as a volume conductor. The potentials are not generated by the olfactory axons projecting through the holes of the cribriform plate, and the exact location of the activated receptor cells within the epithelium can not be deduced from the distribution maps.

In view of these reservations it is reasonable to presume that the different parts of the olfactory epithelium project mainly to nearby parts of the cribriform plate. For instance, a response in the dorsal area of the cribriform plate is most likely caused by activation of receptors mainly located in the roof of the nasal cavity or on the upper turbinates. Adrian suggested that the spatial differences in the bulbar responses to different odors were due to non-homogeneous distribution of olfactory receptors of different specificity. The authors assumed that the neuronal projection from the olfactory mucosa to the olfactory bulb was more or less somatotopic as in fact was demonstrated the same year by Clark (1951). Spatial differences in the distribution of different olfactory receptors were confirmed by Mustaparta in 1971 as evident in the frog.

Stability of the distribution maps

In the present study several types of control experiments were performed, the variation of the number of responses N at the same position for different odor intensities, the stability of one preparation throughout the experiment, and the reproducibility from one animal to another. The results of these control experiments demonstrate the consistency of the reported data. They also include further repetitions of the experiments at homologous positions in different animals. Oxidation of the odorous substances is another possible cause of error. Aldehydes are susceptible to oxidation producing the corresponding acids, the effect of which has distorted the response distribution maps of the three aldehydes. The cyclononanone is known to contain impurities, presumably di-ketones and aldehydes. It is therefore interesting to note that cyclononanone, as the only one of the cycloketones, places itself in a group of substances which also includes the three aldehydes.

Conclusions

The present results show that receptor neurones of different spectra of sensitivity are differentially distributed in the mammalian olfactory mucosa as well. In spite of the complex organization of this epithelium, the spatial distribution of receptor responses is clear. None of

random variation in the electrical noise of the recording devices and the uncertainty in reading of the potentials.

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In spite of these reservations it is reasonable to presume that the different parts of the olfactory epithelium project mainly to nearby parts of the cribriform plate. For instance, a strong response in the dorsal area of the cribriform plate is most likely caused by activation of receptors mainly located in the roof of the nasal cavity or on the upper turbinates. Adrian (1951) suggested that the spatial differences in the buibar responses to different odours were due to a non-homogeneous distribution of olfactory receptors of different specificity. The interpretation assumed that the neuronal projection from the olfactory mucosa to the olfactory bulb was more or less somatotopic as in fact was demonstrated the same year by Le Gros Clark (1951). Spatial differences in the distribution of different olfactory receptors were confirmed by Mustaparta in 1971 as evident in the frog.

The reliability of the distribution maps

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Contamination of the odorous substances is another possible cause of error. Aldehydes in general are susceptible to oxidation producing the corresponding acids, the effect of which may have distorted the response distribution maps of the three aldehydes. The cyclononanone sample is known to contain impurities, presumably di-ketones and aldehydes. It is therefore interesting to note that cyclononanone, as the only one of the cycloketones, places itself in the group of substances which also includes the three aldehydes.

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The present results show that receptor neurones of different spectra of sensitivity are differentially distributed in the mammalian olfactory mucosa as well. In spite of the complex folding of this epithelium, the spatial distribution of receptor responses is clear. None is

the response distribution maps were identical. On the basis of their shape, the maps may be grouped, as belonging to a small number of patterns, some of which make up pairs of almost complementary response distributions. A further analysis of the data on the similarity of response distributions will appear in a subsequent article. Difference in the distribution of the EOG does not indicate that there are discrete areas of uniform specificity. If a large number of receptor types exists, the receptors most likely cover overlapping fields in the olfactory mucosa.

The non-homogeneous distribution of olfactory receptors of different specificity implies that if two substances show different response distributions, they most likely are perceived by the animal as being different. The reverse statement is not necessarily true. Receptors sensitive to different odours may be intermingled so as to produce the same EOG distribution.

This study has been supported by the Norwegian Research Council for Science and the Humanities.

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Effects of Acetylsalicylate on Alkalinization, Acid Secretion and Electrogenic Properties in the Isolated Gastric Mucosa

By

ANDREW GARNER

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Abstract

DICK, A. Effects of acetylsalicylate on alkalinization, acid secretion and electrogenic properties in the isolated gastric mucosa, *Acta physiol. scand.* 1977 99 281-291

Effects of acetylsalicylate (ASA) on the *in vitro* secretory and electrical properties of *Hectera* and *Rana* gastric mucosa have been studied. The gastric mucosa exhibited the lateral surface, while the fundus it is likely that acidification and alkalinization occur simultaneously and that net secretion is the dominance of one or other of these processes. The histamine H_2 receptor antagonist Metoclopramide used to inhibit acid secretion for studies on fundus alkalinization in *Rana temporaria*. Submucosal infusions of 3 mM ASA for 30 min markedly reduced alkalinization in the antrum and the frog fundus. Following removal of ASA there was only partial recovery of this secretion. The drug caused slight inhibition of spontaneous acid secretion in *Nervus* fundus but not of histamine-stimulated acid secretion in the *ig* fundus. Following salicylate removal, the rate of acid secretion increased to a higher level than before stimulation in both tissues. There was a small flux of ASA across the mucosa which was greatest in the *ig* secreting frog fundus ($4.38 \mu\text{mol cm}^{-2} \text{ min}^{-1}$) and least in the antrum ($2.19 \mu\text{mol cm}^{-2} \text{ min}^{-1}$). Exposure of the gastric mucosa to ASA was generally associated with a fall in transmembrane electric potential difference and short-circuit current together with an increase in electrical resistance. It is proposed that the greater recovery of alkalinization to ASA is responsible for the apparent increase in the rate of acid secretion which occurred.

Key words: Gastric mucosa, acetylsalicylate, alkalinization, acid secretion, electrogenic properties

Numerous clinical and laboratory studies have demonstrated that aspirin damages the gastric mucosa. A single oral dose can cause visible erosion of the epithelial lining of the stomach (Anderson 1964, Thorsen *et al.* 1968), an increase in the rate of exfoliation of gastric mucosal cells (Croft 1966) and gastric bleeding (Grossman, Matsumoto and Lichter 1961, Davenport 1969). Exposure of the mucosa to aspirin is also associated with changes in electrophysiological properties and permeability characteristics, both *in vitro* (Davenport 1964, Framming 1971, Smith *et al.* 1971) and *in vivo* (Becha, Hirschowitz and Shoemaker 1972, Flennstrom and Maraden 1973).

Assessment of the effects of aspirin on gastric acid secretion *in vivo* is complicated by the fact that inhibition of acid output *per se* is accompanied by an increase in mucosal perme-

the response distribution maps were identical. On the basis of their shape, the maps can be grouped, as belonging to a small number of patterns, some of which make up pairs of almost complementary response distributions. A further analysis of the data on the similarity of response distributions will appear in a subsequent article. Difference in the distribution of the EOG does not indicate that there are discrete areas of uniform specificity. If a large number of receptor types exists, the receptors most likely cover overlapping fields in the olfactory mucosa.

The non-homogeneous distribution of olfactory receptors of different specificity implies that if two substances show different response distributions, they most likely are perceived by the animal as being different. The reverse statement is not necessarily true. Receptors sensitive to different odours may be intermingled so as to produce the same EOG distribution.

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1. *Necurus* beryllate (10^{-4} M) and histamine ($0.6 \cdot 10^{-4}$ M) to the nutrient solution together with continuous release of histamine at rate of $1 \cdot 10^{-4}$ mol min $^{-1}$. All solutions were adjusted to the same calculated osmolarity as the nutrient solution described above, by altering the NaCl content.
2. The effect of inorganic (Sigma Chemical Co.) as determined by substitution of the nutrient side solution in a similar way containing approximately 3 mM ASA, during the second period (30-60 min) of the experiment exposure to the drug, both the secretory and nutrient side solutions were removed and replaced with alk. The transmembrane flux of ASA was determined from the total electrolyte concentration in the secretory solution, measured spectrophotometrically at 301 nm (Carl Zeiss PMQ 11 spectrophotometer) following 15 min in 3-trichloroacetic acid for 30 min at 90°C.

Results

Characteristics of data

Mean values for PD, R and I_{sc} during each 30 min period and the mean secretory rate during the 15 min periods, were obtained for individual expts. The overall mean \pm one standard error (S.E.) for each experimental group is presented. Statistical significance was determined using Student's "t" test, by comparing data during the last 4 periods (30-150 min) (i.e. during and after administration of ASA, with that from the first 30 min period.

There are no significant differences ($p > 0.10$) among electrical parameters and secretory rates in any of the 4 control groups during the different time periods. The coefficients of variation were large, but this was due to differences between individual mucosae. In the controls, variation within each expt. was low reflecting the good stability conditions obtained during the first 3-4 h after isolation of the tissues.

Alkalinization in *Necurus* antrum

The electrophysiological properties of *Necurus* gastric antrum are shown in Table 1. This tissue developed small PD and low I_{sc} compared with the fundic mucosal preparations. In addition, spontaneous alkalinization of the luminal side occurred with a mean secretory rate of $0.34 \mu\text{eq cm}^{-2} \text{ h}^{-1}$. Exposure of the nutrient side to 3 mM ASA for 30 min resulted in a significant decrease in transmembrane PD (0.02 $p < 0.05$). When the drug was removed the PD continued to decline and there was also a fall in I_{sc} ($p = 0.01$ for both). However, there was no significant change in R ($p = 0.10$), either during or after exposure to the drug. The effect of ASA on spontaneous alkalinization is shown in Fig. 1. When ASA was present in the nutrient solution, secretion ceased in 4 out of 7 mucosae and was greatly

TABLE 1. Electrical properties of control and ASA-treated *Necurus* antral mucosae during spontaneous alkalinization. ASA (3 mM) was applied to the nutrient side during the second period of the expt (30-60 min). Mean values \pm S.E. are given (number of expts.).

		0-30 min	30-60 min	60-90 min	90-120 min	120-150 min
Control (n = 5)	PD (mV)	3.14 ± 0.55	3.27 ± 0.64	3.38 ± 0.72	3.31 ± 0.78	3.17 ± 0.73
	R ($\Omega \text{ cm}^2$)	441 ± 52	436 ± 53	444 ± 53	449 ± 54	471 ± 120
	I_{sc} ($\mu\text{eq cm}^{-2} \text{ h}^{-1}$)	0.33 ± 0.06	0.39 ± 0.08	0.45 ± 0.09	0.41 ± 0.10	0.39 ± 0.14
			ASA			
ASA-treated (n = 7)	PD (mV)	6.68 ± 0.58	5.53 ± 0.64	4.33 ± 0.49	3.57 ± 0.32	2.96 ± 0.28
	R ($\Omega \text{ cm}^2$)	427 ± 39	392 ± 27	387 ± 33	411 ± 57	414 ± 40
	I_{sc} ($\mu\text{eq cm}^{-2} \text{ h}^{-1}$)	0.49 ± 0.05	0.43 ± 0.05	0.35 ± 0.05	0.27 ± 0.04	0.23 ± 0.04

bility which results in a loss of luminal H^+ by back-diffusion into the mucosa (Dave 1967). There is usually a decrease in acid secretion *in vitro* (Flemström and Mårds Kaskbæk 1973) although the fact that only net flux is measured may be responsible for some of the conflicting reports concerning the influence of aspirin on H^+ secretion (Davenport 1964, Lynch, Shaw and Milton 1964).

Recent *in vitro* studies have shown that the surface epithelial cells of the antrum of *Necturus* (Flemström and Sachs 1975) and the fundic mucosa of *Rana temporaria* (Flemström 1976), in which acid secretion was inhibited with the histamine H_2 receptor antagonist Burimamide, produced an alkaline secretion which was sensitive to inhibitors of metabolism and to acetazolamide. The role of an active alkalization may be to protect the antral luminal surface from exposure to a high concentration of H^+ and it might be one mechanism neutralizing the acid gastric contents. The effect of drugs on the pH of alkalization may further complicate the measurement of gastric acid secretion: normally masks the simultaneous but quantitatively smaller alkaline secretion.

It was of interest therefore, to examine the effects of aspirin on the electrical properties and processes of acidification or alkalization in isolated gastric mucosae from *temporaria* and *Necturus* in order to further study the mechanism by which salicylates induce gastric secretion. In addition, this approach provides information on the mechanism of salicylate-induced gastric damage in terms of the relative sensitivity of different secretory mucosal cell types to the drug.

Methods

Necturus were purchased from Mogul-Ed, Wisconsin, USA and *Rana temporaria* from Firma Laubingen-Doorn, Germany. The animals were kept in tap water at $10^\circ C$ for up to 6 weeks during time they were force-fed with 100–200 g of liver once a week. No food was given during the two preceding an experiment.

Animals were killed by section of the spinal cord, the abdomen opened and the stomach removed. The mucosa was separated from the rest of the stomach wall by blunt dissection in frog-Ringer solution mounted as a membrane between the two halves of a Perspex chamber. The exposed mucosal surface was 1.8 cm^2 . In the case of *Rana temporaria* only fundic mucosae were used. With *Necturus* separate experiments were performed on mucosae from both the antral and fundic parts of the stomach. The antrum is readily distinguished by its paler appearance and the absence of folds. Both sides of the mucosa were bathed with 20 ml of solution circulated by means of a gas lift. The nutrient (serosal) side solution was gas-saturated with 95% O_2 –5% CO_2 and the secretory (luminal) side solution with 100% O_2 , prewashed in $NaOH$ to remove contamination with CO_2 . The nutrient solution had the following composition: 81.6 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.8 mM KH_2PO_4 , 17.8 mM $NaHCO_3$, 2.0 mM Glucose, pH 7.4. A similar but unbuffered solution was used on the secretory side and the pH kept constant at 7.40 by addition of HCl ($5 \times 10^{-3} \text{ M}$) or NaOH ($15 \times 10^{-3} \text{ M}$) under automatic control from a pH-stat test (Radiometer Copenhagen). The rates of alkalization or acid secretion were calculated from the amount of HCl or NaOH infused.

The transmucosal potential difference (PD) was measured via 2 matched calomel electrodes and recorded with a high-input impedance voltmeter. Short-circuit current (I_{sc}) was applied from an external source. Ag-AgCl electrodes separated from the circulating solution by thick cellophane membranes. The pH was adjusted manually and calculated from the voltage drop over a precision resistor. The electrical resistance (R) was determined from the change in potential immediately following passage of a fixed current ($30 \mu A \text{ cm}^{-2}$) through the tissue in either direction.

Electrical parameters and secretory rate were measured every 5 min during 3 consecutive 30 min periods. 4 experimental groups, based on secretory differences, were used. In *Necturus* mucosae, alkaline (antrum) and acid secretion (fundus) were spontaneous. Alkalization in *Rana temporaria* fundus induced by addition of Metiamide (10^{-3} M) to the nutrient side solution, and acid secretion by add-

TABLE II Electrical properties of control and ASA-treated *Rana temporaria* fundic mucosae during Metiamide-induced alkalinization. ASA (3 mM) was applied to the mucosal side during the second period of the expt. (30-60 min). Mean values \pm S.E. are given (n = number of expts.).

		0-30 min	30-60 min	60-90 min	90-120 min	120-150 min
Control	PD (mV)	14.60 \pm 2.83	14.15 \pm 2.74	13.67 \pm 2.60	13.34 \pm 2.59	13.08 \pm 2.53
	R (Ω cm ²)	536 \pm 104	523 \pm 89	528 \pm 91	533 \pm 97	532 \pm 101
	I_{sc} (μ eq cm ⁻² h ⁻¹)	0.97 \pm 0.15	0.94 \pm 0.16	0.92 \pm 0.16	0.90 \pm 0.16	0.88 \pm 0.15
		ASA				
ASA-treated	PD (mV)	15.38 \pm 2.71	10.69 \pm 2.54	11.90 \pm 2.48	12.27 \pm 2.28	11.93 \pm 2.22
	R (Ω cm ²)	334 \pm 83	363 \pm 82	347 \pm 84	345 \pm 84	345 \pm 89
	I_{sc} (μ eq cm ⁻² h ⁻¹)	1.84 \pm 0.40	1.22 \pm 0.33	1.42 \pm 0.32	1.44 \pm 0.30	1.40 \pm 0.29

alkalinization of the secretory side following the disappearance of spontaneous acid secretion (Kornstein 1976).

The electrical properties and secretory rates in control and ASA-treated fundic mucosae on *Rana temporaria* in which alkalinization was induced with the histamine H₂ receptor antagonist Metiamide (10⁻⁶ M) are shown in Table II and Fig. 2. The results reported are on mucosae in which a steady rate of alkalinization was recorded within 2 h (mean = 5 min), which occurred in 13 out of a total of 21 preparations. Metiamide-treated mucosae had a lower PD and I_{sc} and a higher R than acid secreting mucosae from the same species (Table IV). The rate of alkaline secretion in the control group ($\sim 0.46 \mu\text{eq cm}^{-2} \text{h}^{-1}$) was similar to that in the *Necturus* stratum ($\sim 0.38 \mu\text{eq cm}^{-2} \text{h}^{-1}$). Addition of ASA (3 mM) resulted in a significant fall in PD and I_{sc} ($0.02 < p < 0.05$ for both) but no change in R. After removal of the drug, the electrical parameters were not significantly different ($p > 0.10$) from before administration. There was a reversal in secretory response during the period

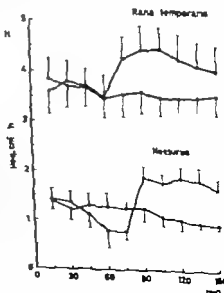


Fig. 3 Rates of spontaneous acid secretion (*Necturus*) and histamine-stimulated acid secretion (*Rana temporaria*) in control and ASA-treated fundic mucosae. ASA (3 mM) was applied to the mucosal side of the tissue for 30 min during the second period of the expt. (30-60 min). Mean values \pm S.E. are given. Control (open circles), 4 for *Necturus* and 5 for *Rana temporaria* and ASA-treated (filled circles), 6 for *Necturus* and 7 for *Rana temporaria*.

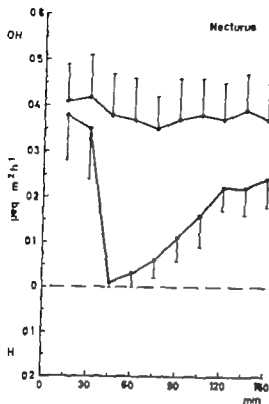


Fig. 1

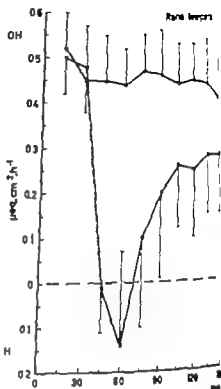


Fig. 2

Fig. 1. Rates of spontaneous alkalinization in control and ASA treated *Necturus* antral mucosa. ASA (3 mM) was applied to the nutrient side of the tissue for 30 min during the second period of the experiment (30–60 min). Mean values \pm S.E. are given. Control (open circles, $n=5$) and ASA treated (filled circles, $n=7$).

Fig. 2. Rates of Metiamide-induced alkalinization in control and ASA treated *Rana temporaria* fundic mucosa. ASA (3 mM) was applied to the nutrient side of the tissue for 30 min during the second period of the experiment (30–60 min). Mean values \pm S.E. are given. Control (open circles, $n=5$) and ASA-treated (filled circles, $n=8$).

reduced in the remainder ($p < 0.001$). There was a partial recovery in response following wash-out of the drug but the rate was less than before administration ($p < 0.01$). When 3 mM ASA was applied to the secretory side ($n=2$), alkalinization during the period 60–150 min was totally abolished. In this case, however, it was not possible to titrate secretion while the drug was present during which time the pH of the solution rose by 0.7 units. The antral mucosa has no acid secretory capacity as evidenced by the absence of acid secretion after challenge with histamine (10^{-4} M) and in this tissue, unlike *Rana temporaria* fundus (see Fig. 2), cessation of alkalinization was not associated with an onset of acid secretion.

Alkalinization in *Rana temporaria* fundus

The gastric mucosa from most amphibian species *in vitro* secretes acid spontaneously (Heben 1965) due largely to stimulation of acid secreting cells by endogenous histamine released from mucosal stores (Kasbekar 1967; Rangachari 1975). Addition of the histamine antagonist Burimamide to the nutrient side of isolated fundic mucosae is associated with a

Transmucosal flux of ASA in *Necturus* and *Rana temporaria* isolated gastric mucosae. The nutrient side of the tissue was exposed to ASA for 30 min and the bidirectional flux calculated from the concentration in the secretory side solution. Drug concentration as determined as total salicylate. Mean values \pm S.E. are given (—number of expts.).

	Secretory	Initial concentration on nutrient side (mM)	Final concentration on secretory side (mM)	Flux ($\mu\text{mol cm}^{-2} \text{ min}^{-1}$)
<i>Necturus</i> (n = 7)	Alkalinization	3.02 ± 0.14	0.12 ± 0.02	2.19 ± 0.37
<i>Rana temporaria</i> (n = 8)	Alkalinization	3.09 ± 0.13	0.19 ± 0.04	3.57 ± 0.69
<i>Necturus</i> (n = 8)	Acidification	3.00 ± 0.06	0.20 ± 0.01	3.73 ± 0.23
<i>Rana temporaria</i> (n = 6)	Acidification	2.84 ± 0.13	0.4 ± 0.01	4.38 ± 0.21

extended by Flemström (1971). In this tissue, PD and I_{sc} were higher and R lower ($p < 0.02$ for all) than in the same species during Metiamide-induced alkalization or during fundus during spontaneous acid secretion. There was no significant change in the electrical parameters ($p > 0.10$) following exposure of the mucosa to ASA (Table I). The PD tended to fall by 3–4 mV throughout the course of the expt., but this also occurred in the control group. The mean rate of acid secretion was $3.6 \mu\text{eq cm}^{-2} \text{ h}^{-1}$ and remained unchanged in the presence of ASA (Fig. 3). On removal of the drug the rate of secretion increased, as also found in the spontaneously secreting *Necturus* fundus, but this effect was only of low significance ($0.05 > p > 0.10$).

Mucosal flux of ASA

In the present expts. ASA (pK_a 3.6) was added to the buffered nutrient side solution at pH 7.2 and was therefore present in the ionized form. The flux of ASA from the nutrient to the secretory side of the membrane was determined as total salicylate and is shown in Table V. Mucosal flux was greatest in the acid secreting *Rana temporaria* fundus ($4.38 \mu\text{mol cm}^{-2} \text{ min}^{-1}$), where the secretory side concentration after 30 min amounted to 8.45% of the administered dose. This tissue also had the lowest recorded mucosal resistance. However, cyclic flux was not significantly different ($p > 0.05$) than that in the same tissue during alkalization or in *Necturus* fundus during spontaneous acid secretion. The lowest flux occurred in the *Necturus* antrum ($2.19 \mu\text{mol cm}^{-2} \text{ h}^{-1}$) and was significantly less ($0.02 < p < 0.05$) than in all other tissues.

Discussion

Gastric physiologists have been mainly concerned with the acid secreting fundic mucosa and only recently have investigations been directed at the antral region of the stomach. The electrophysiological properties of *Necturus* antrum were characterized by Flemström and Sachs (1975), who reported that this tissue generated a low PD (4–10 mV) and I_{sc} ($\sim 0.41 \mu\text{eq cm}^{-2} \text{ h}^{-1}$) dependant on mucosal to serosal Na⁺ transport, but had a relatively high resist-

TABLE III Electrical properties of control and ASA treated *Necturus fundic* mucosa during spontaneous acid secretion. ASA (3 mM) was applied to the nutrient side during the second period of the expt. (30–60 min). Mean values \pm S.E. are given (n = number of expts.).

		0–30 min	30–60 min	60–90 min	90–120 min	120–150
Control (n = 4)	PD (mV)	16.02 \pm 1.90	16.33 \pm 1.44	15.85 \pm 1.29	15.30 \pm 1.04	15.13 \pm 1.11
	R (Ω cm ²)	500 \pm 113	480 \pm 102	444 \pm 88	506 \pm 95	516 \pm 98
	I _{ao} (μ eq cm ⁻² h ⁻¹)	1.26 \pm 0.12	1.34 \pm 0.13	1.25 \pm 0.12	1.15 \pm 0.08	1.13 \pm 0.08
ASA						
ASA treated (n = 6)	PD (mV)	15.83 \pm 1.53	14.04 \pm 2.26	17.08 \pm 1.97	15.54 \pm 2.08	15.63 \pm 2.10
	R (Ω cm ²)	470 \pm 46	563 \pm 77	444 \pm 77	490 \pm 78	508 \pm 78
	I _{ao} (μ eq cm ⁻² h ⁻¹)	0.84 \pm 0.16	0.72 \pm 0.19	0.86 \pm 0.26	0.87 \pm 0.26	0.83 \pm 0.26

In which ASA was present in the nutrient side solution and although an alkaline secret appeared when the drug was removed, the rate was less than before administration ($p < 0.05$)

Acidification in *Necturus fundus*

In contrast with mucosa from the antral part of the stomach, the fundic mucosa of *Necturus* secreted acid spontaneously ($\sim 1.2 \mu\text{eq cm}^{-2} \text{ h}^{-1}$) and had a considerably higher PD and greater I_{ao}. However the value of R in the two tissues was similar. The effect of one side ASA (3 mM) on the rate of acid secretion is shown in Fig. 3. There was a slight inhibition of secretion during exposure to the drug and in the first 15 min after its removal ($0.05 < p < 0.1$) following which the rate of secretion increased to a higher level than before ($0.02 < p < 0.05$). The decrease in acid secretion was accompanied by a fall in PD and an increase in R (Table III). There was a small rise in PD and I_{ao} together with a fall in R during the subsequent increase in rate of secretion. However the changes in electrical activity were not significant ($p > 0.10$). There was no change in the rate of acid secretion following secretory side application of 3 mM ASA (n = 2).

Acidification in *Rana temporaria fundus*

Addition of butyrate and continuous infusion of histamine into the nutrient solution used to obtain good long-term stability of acid secretion in the isolated fundic mucosa.

TABLE IV Electrical properties of control and ASA treated *Rana temporaria* fundic mucosa during histamine-stimulated acid secretion. ASA (3 mM) was applied to the nutrient side during the second period of the expt. (30–60 min). Mean values \pm S.E. are given (n = number of expts.).

		0–30 min	30–60 min	60–90 min	90–120 min	120–150
Control (n = 5)	PD (mV)	25.74 \pm 3.52	4.71 \pm 3.58	23.65 \pm 3.51	22.57 \pm 3.38	21.87 \pm 3.38
	R (Ω cm ²)	46 \pm 35	25 \pm 54	275 \pm 60	267 \pm 53	264 \pm 44
	I _{ao} (μ eq cm ⁻² h ⁻¹)	2.43 \pm 0.41	2.40 \pm 0.41	2.34 \pm 0.42	2.28 \pm 0.40	2.26 \pm 0.39
ASA						
ASA treated (n = 7)	PD (mV)	26.39 \pm 3.75	3.65 \pm 3.26	23.05 \pm 3.72	22.20 \pm 3.77	22.73 \pm 4.04
	R (Ω cm ²)	200 \pm 26	240 \pm 19	215 \pm 77	222 \pm 4	217 \pm 11
	I _{ao} (μ eq cm ⁻² h ⁻¹)	3.19 \pm 0.39	2.91 \pm 0.38	3.00 \pm 0.47	2.97 \pm 0.49	3.06 \pm 0.49

ionized form (Flemström and Maraden 1973). Although the drug permeated the gastric mucus, the pK_a value precludes it from contributing to the titratable acidity of the secretory solution. The unchanged rate of acid secretion in *Rana temporaria* fundus in the presence of ASA, which markedly reduced alkalization in the same tissue, is a further indication that the drug caused a simultaneous inhibition of acid secretion.

The rate of acid secretion increased in both species following removal of ASA. This effect is only of low significance in the *Rana temporaria* and was probably due to the fact that any increase is superimposed on the already high rate of histamine-stimulated secretion. The recovery of alkaline secretion in both the antrum and fundus, combined with the fact that ASA inhibited acid output in *Necturus* fundus, makes it unlikely that the rise in acid secretion after ASA was due to actual stimulation. Furthermore, the post-ASA secretory profiles strongly suggest that under the present *in vitro* conditions at least, measured secretory response is a reflection of the balance between acid and alkali output.

The rate of passage of drugs across biological membranes decreases following administration in the ionized form, and the transmucosal flux of ASA in these expts. was low. The smallest flux occurred in the antrum, where the secretory side concentration after 30 min was only 4% of the applied dose. However the mucosal flux was not related to the secretory response, since in this tissue there was an almost total inhibition of alkalization. The drug concentration in the mucosa, and in particular within the secretory cells, is of more relevance. Although not measured in the present study indirect measurement based on the difference between the concentration administered and the sum of that recovered in the secretory and solvent solutions, indicated that mucosal concentration was greatest in the antrum.

The origin of the alkaline secretion from the gastric mucosa is unknown but a number of observations suggest that it is the surface epithelial cell. These cells contain most of the gastric mucosal carbonic anhydrase (Vollrath 1959) and the gastric antrum is composed mainly of this type of cell. Also, ASA was a more effective inhibitor of alkalization when applied to the luminal side of the mucosa than from the serosal side. The weak base ampyrine was found to accumulate in parietal cells (Berglindh, Heklander and Öbrink 1976) suggesting that the cytoplasm of these cells is basic. Conversely it would be expected that ASA could accumulate preferentially in the surface epithelial cells and this may be responsible for the greater sensitivity of alkalization compared with acid secretion to the inhibitory actions of the drug. The present results support the electron microscopical observations of Hagson and Ito (1971), who reported that the surface epithelial cells of mouse gastric mucosa were particularly susceptible to the damaging effects of ASA, but concluded that the action of the drug was not directed at secretory function in particular. However the drug was administered in a higher dose than in the present study and from the luminal side in the ionized form. Under these conditions it is to be expected that a considerably higher intracellular concentration would be achieved and that general cellular damage would result. The present study suggests that a much lower concentration is required in order to affect the secretory function of the mucosa and furthermore that this function is extremely sensitive to ASA.

In the amphibian gastric mucosa *in vitro* alkalization is considerably more sensitive than acid secretion to the effects of ASA. Inhibition of alkalization is associated with an

ance. There was also an active alkalization of the luminal surface. Results from the present study are in good agreement with their data in respect to electrical parameters. In addition the tissue was found to secrete alkali spontaneously at a steady rate which amounted to 1/3rd of the rate of spontaneous acid secretion by the fundic mucosa from the same species.

Quantitation of conductance pathways in the antrum have shown that it is a "tight" epithelium in which transport is due mainly to the extracellular passage of ions (Sprengel *et al* 1975). Alkalization may be of importance, therefore, in protecting the mucosa from the large H^+ gradient generated by the adjacent fundus, which is a "tight" epithelium (Sprengel, Shoemaker and Sachs 1974). The high shunt conductance may also be of relevance to the transmucosal flux of weak electrolytes such as ASA, since there is evidence that drug in the ionized form may permeate biological membranes via a paracellular route (Chang *et al* 1970, Wright and Pietras 1973).

The gastric antrum has no acid secretory capacity as gauged by the absence of an effect with histamine. Furthermore, the abolition of alkalization in the antrum following ASA administration was not followed by the appearance of acid secretion. The assessment of fundic secretion, however, is complicated by the fact that this tissue has the ability to secrete both acid and alkali. Since it is not possible to titrate acid and alkali output simultaneously the measured (net) secretion is only a reflection of the dominance of one or other of the processes. The spontaneous secretion in *Necturus* fundus was always acid, although this tissue does secrete alkali when acid secretion is inhibited with Burimamide (Flemström 1976). In *Rana temporaria* fundus, spontaneous alkalization occurred in 4 out of 13 mucosae from the start of the expt. and the remainder had an alkalization revealed following inhibition of acid secretion with Metiamide. This drug was used in preference to Burimamide which induces alkalization in only 60% of cases (Flemström, personal communication).

Alkalization is an active process which is inhibited by antimetabolites and acetazolamide (Flemström 1976). Acetazolamide has been demonstrated to cause gastric mucosal erosion in the dog (Werther, Hollander and Altamirano 1965) and may act by reducing the formation of bicarbonate and consequently the degree of neutralization of the gastric content. In the present study ASA, which is well known to cause gastric mucosal damage, produced a marked inhibition of both spontaneous and Metiamide-induced alkalization. This observation itself makes it unlikely that alkalization is due to the passive permeation of bicarbonate from the nutrient solution since administration of ASA was associated with an increase in mucosal conductance. In addition Metiamide which was used to initiate alkalinization in the frog, also caused an increase in conductance. An increase in the luminal bicarbonate concentration occurred *in vivo* following oral administration of 20 mmol ASA in the unionized form (Davenport 1966) and when the interstitial pressure of the gastric mucosa was increased (Altamirano, Requena and Pérez 1975). However, in these cases it is likely that there is an ultrafiltration of bicarbonate resulting from an increase in the permeability of the paracellular pathways of the mucosa.

The decrease in alkalization in *Rana temporaria* fundus could conceivably result from stimulation of acid secretion by ASA. This is unlikely since the drug inhibited acid secretion during the period of administration in *Necturus* fundus and has also been demonstrated to cause inhibition of secretion in *Rana temporaria* fundus when applied to the luminal side.

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inability of the mucosa to withstand a low intraluminal pH (Flemström 1976). The apparent increase in acid secretion is probably due to a reduction in the degree of neutralisation in the lumen. This effect, combined with the increase in mucosal permeability may be responsible for the gastric damage associated with ASA administration which occurs particularly in the antral region of the stomach which is a common site of ulceration in man.

Thus in addition to inhibition of parietal secretion *per se* and the loss of H⁺ by back-diffusion an important consideration in the assessment of gastric secretion should be the relative contributions of acid and alkaline output. In fact the effect of drugs on the phenomena of neutralisation may be more significant than an effect on back-diffusion, as mucosal conductance was unaffected or marginally increased after ASA and quickly recovered on removal of the drug, whereas secretory response showed only a slow rate of recovery.

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cell bodies in the geniculate ganglion outside the CNS while the efferent fibres have cell bodies in the CNS (van Burskirk 1945, Foley and DuBois 1943, Foley 1945, Villiger, Woodreau et al. 1971). Therefore, section of the facial root between the CNS and the ganglion ought to cause degeneration of the efferent fibres while the sensory remain. In the present study this section has been made in rats. The paper will give a first report of some of the results obtained.

Methods

Experiments to be described were done on adult male Sprague-Dawley rats weighing 300 ± 30 g. The surgery was performed under a combination of 2 anaesthetic drugs. The anaesthesia has recently been described (Hellekant and Gopel 1975) and will only be reported briefly here. The two drugs used were Hypnorm and Mebumal.[†] The proportion in which the drugs were used was adapted to the experimental needs. Less Mebumal than Hypnorm was used when the facial root was cut and more during the final experiments when the tongue was perfused.

The surgery for cutting the facial root was as follows: The skin on the head was divided by a sagittal cut between rostrally to the occipital crest. The tissue overlying the interparietal bone was divided with a scalpel and the bone exposed. A 3–4 mm piece of the bone from the right lateral border to about 1 mm on the other side of the midline was removed. The meninges were divided to expose the cerebellum. The cerebellum was gently retracted from the right side of the cranial cavity until the root of the facial nerve was exposed. The root was then transected. Care was taken not to damage the VIII nerve which enters rostrally and somewhat posterior to the facial nerve. The wound was then closed and sutured in two or three parts. The piece of bone was not returned. After surgery 10 ml Anidolol glucose[‡] and 0.2 ml atropine were given i.c. Once the technique was developed only one out of 30 rats died as a direct result of surgery.

After intervals ranging from 1 week to 3 months the chorda tympani proper nerve outside the tympanic membrane was dissected. The nerve was cut and put on electrodes to allow recording from and electrical stimulation of its peripheral part. The vascular resistance of the tongue or the temperature of its surface or both were recorded. The technique for studying the vascular resistance of the tongue was described earlier (Hellekant 1971 b) but, as it has been slightly modified, it will be repeated here.

The tongue was continuously perfused at a constant flow rate while the perfusion pressure was recorded. The perfusion loop consisted of 2 polythene tubes connected via two 3-way cocks with an intermediate latex rubber tube which was placed in a peristaltic pump, Hobart RD 074. The loop had a volume of 1 ml. The cocks made it possible to direct the flow either to the tongue or to femoral veins and to flush the system with Ringer-Heparin[§] solution. During the perfusion blood was taken from the right femoral artery lateral through the loop by the pump and ejected through the right external maxillary artery into the external carotid artery from which the lingual artery branches. Thus the tongue could be supplied with blood in the normal way until perfusion started. Then the pump was started and the external carotid artery was clamped between the heart and the branching of the lingual artery. Other arterial branches were partially occluded. This was also the case with the branch which is about one-third of the rate is given off from the lingual artery close to its origin and which supplies the surrounding tissue and not the corpus of the tongue. The perfusion pressure was monitored in the right posterior auricular artery close to its surface to the external carotid artery. The contralateral blood supply to the tongue was not touched. This seemed to be justified because each half of the tongue is largely supplied with blood from the ipsilateral lingual artery (Hellekant 1971).

The temperature of the surface of the tongue was measured by a thermocouple attached to the tongue with a suture so that the temperature difference between the right and left dorsal sides of the tongue was recorded. The thermocouple was connected to a DC amplifier, whose output was recorded. The systemic blood pressure was recorded in the right femoral artery. Electrical stimulation was delivered from Grass stimulator (S52). The stimuli were pulses with a duration of 2 ms and a current of 10–20 μ A. Their frequency varied from 1–100 Hz.

Results

The records shown in Fig. 1 were obtained during perfusion of the tongue in a normal rat. The top trace records the blood pressure to the tongue. The second trace shows the systemic

Vasodilator Fibres to the Tongue in the Chorda Tympani Proper Nerve

By

GÖRAN HELLEKANT

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Abstract

HELLEKANT G *Vasodilator fibres to the tongue in the chorda tympani proper nerve* Acta physiol. scand. 1977 99 292-299

Electrical stimulation of the chorda tympani proper nerve (CT) causes vasodilatation in the constant flow perfused tongue. It has been suggested as an explanation for this, that the vasodilatation is the result of antidromic impulses in sensory fibres of the CT. However after degeneration of the efferent fibres in the CT in the rat it was found that electrical stimulation of the CT caused no vasodilatation. Since, by all criteria available, the sensory fibres remained, the result indicates that antidromic impulses could not be the cause of the vasodilatation observed in tongues with intact nerve supply. Further since there are no glands in the part of the tongue innervated by the CT and the circulation of the large salivary glands is separate from the tongue in the rat, it is concluded that the vasodilatation was not a secondary effect of glandular activity but was caused by vasodilator fibres in the CT.

Ever since Claude Bernard (1858) described how electrical stimulation of the chorda tympani proper nerve, CT, produced vasodilatation in the submandibular salivary gland, the question has puzzled physiologists whether it, in addition to its salivary and afferent fibres, contains specific vasodilator fibres. Though the evidence for specific vasodilator fibres in the nerve to the submandibular salivary glands is growing (Gautvik 1970) it has not been possible to prove conclusively that this vasodilatation is not a secondary effect of the secretion caused. A similar difficulty exists with regard to the vasodilatation in the tongue. Though there are no glands in the anterior part of the tongue, it can always be claimed that the vasodilatation observed was the result of antidromic impulses in the sensory fibres of the CT nerve (Eric and Uvnäs 1952, Eccles and Wallis 1974). We have earlier observed and described changes in the efferent activity of the CT which indicate that it contains vascular fibres (Hellekant 1972). We have also recorded changes of the vascular resistance of the tongue to stimulation of this nerve (Hellekant 1970) but we have not been able to rule out the argument presented above.

However we have now developed a technique which seems to cause degeneration of all efferent fibres in the nerve without destroying its sensory portion. The sensory fibres have

in vascular resistance, blood pressure and in periods were recorded as in the previous case in an animal whose right facial root had been cut. The record shows that stimulation of the chorda did not elicit decrease of vascular resistance of the tongue though stronger stimulation at 40 and 70 Hz.



was in contrast to the results obtained in rats which had their facial root cut. Fig. 3 records from an experiment in such a rat. The same procedure was applied as in Fig. 1 but pulse rates were higher 40 and 70 Hz. It is seen in Fig. 3 that in spite of this strong stimulation no decrease of vascular resistance was recorded.

These experiments were repeated in 15 rats of which every second animal had its facial root cut. Results were similar. However in 4 of the animals with cut facial root a slight decrease in perfusion pressure was traced at stimulus rates above 40 Hz. Quantification of these effects was difficult, but because any effects along these lines after the facial root was cut were of importance, it was tried. The decrease observed in one of these animals was estimated at maximum 10% in the other 5%. It is evident that these effects were small compared with those recorded in a normal animal, Fig. 1 and Fig. 2. In fact the decreases were difficult to observe. Similar small effects could also be observed in these animals during strong stimulation (40 Hz) of their trigeminal proper nerves.

In conclusion, it seems that there was a basic difference between the normal animals and those with cut facial nerves. Only in animals in which the efferent fibres in their chorda tympani nerve remained could a substantial decrease of vascular resistance of the tongue be recorded. The effects in the "cut" animals were only observed during strong unphysiological stimulation (40 Hz). It may be of interest that they could also be elicited by stimulation of the trigeminal proper nerves too.

During stimulation in unperfused normal rats it was observed that the tongue on the stimulated side darkened indicating hyperemia on that side. In the unperfused normal rat where blood to the tongue was not cooled by the perfusion loop, it seemed reasonable that the temperature of the tongue should change during stimulation, as the result of the hyperemia. To test this, a thermocouple was attached to the tongue, in such a way that a possible difference in temperature between the right and left sides could be recorded. The method is described under Methods (p. 293). It was then observed that the temperature increased on the side where the CT nerve was stimulated. This was not observed after the facial root had been cut. Further in 12 rats both vascular resistance and tongue temperature were recorded. Thus we recorded the vascular resistance by perfusing during the stimulation, switched then to normal circulation and repeated the stimulation, while we recorded the temperature difference between the right and the left sides of the tongue. It was then observed that an increase of temperature of the tongue never occurred as a result of chorda tympani stimulation if a decreased vascular resistance could not be recorded.



Fig. 1. In the top trace the vascular resistance the tongue was recorded during perfusion in normal rat. The middle trace records the systolic arterial blood pressure and the lower trace is signal indicating two periods of electrical stimulation of the right CT with pulses at 7 and 40 Hz, 20 μ A.

blood pressure in one femoral artery. The bottom trace is a signal indicating the periods of electrical stimulation of the peripheral part of the chorda tympani proper nerve (CT). The perfusion pump was first started. Then the blood supply from the heart was stopped by occlusion of the common carotid artery between the heart and the branching of the lingual artery so that the tongue was supplied with blood from the pump. The output of the pump was adjusted to give a flow to the tongue which, judged by the blood pressure, was approximately the same as before the occlusion. Then during the constant perfusion the CT was stimulated. During the first stimulation the pulses were delivered at 7 Hz and during the second at 40 Hz. The current was 20 μ A. The record shows that the first stimulation caused moderate vasodilatation while the second gave a strong dilatation in the tongue. The duration and shape of decrease in vascular resistance that was recorded during the stimulation is of interest. Periods of stimulation larger than 1 min were not tried in this series of experiments. The records indicate that the vasodilatation reached a plateau and remained there during stronger stimulation while a slight recovery was observed during less strong stimulation.

Fig. 2 presents in diagrammatic form several observations on the relationship between the changes of the vascular resistance observed and the frequency of stimulation applied during constant-flow perfusion of the tongue in normal animals. The average resistance before stimulation was considered as 100. Then the change observed during the stimulation was measured and expressed as a percentage of the value before stimulation. In Fig. 2 values from 8 rats are included, but only the stimulus frequency of 40 Hz was applied in all the rats. Otherwise each point comprises data from 3–4 rats. The bars give the SD. The diagram is not intended as an exact quantitative description of the stimulus/effect relationship. However, certain conclusions can be drawn from the diagram. Thus it seems that large decreases in vascular resistance than 50% could not be elicited by CT stimulation. Further, it suggests that even a stimulus frequency of 1 Hz was able to affect the vascular resistance of the tongue. Finally it shows that the largest vascular changes occurred at changes in the low stimulus frequency range (1–4 Hz) and that maximum effects were obtained at < 20 Hz.

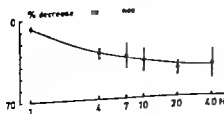


Fig. 2. The decrease of vascular resistance in the tongue of eight normal rats has been plotted against the stimulus frequencies applied to the CT nerves. The bars illustrate the SD. The diagram shows that the largest changes were elicited by changes in the lowest frequency range and that a plateau was reached between 10–20 Hz, after which further increase of stimulus frequency did not cause more vasodilatation.

modalities and efferent fibres to salivary glands and perhaps to other structures as also (Hellekant 1971). A number of anatomists have investigated the facial nerve (DuBois 1943, Baskirk 1945, Foley 1945, Villiger 1964, Boudreau *et al* 1964). Information available suggests that the sensory fibres of the CT have their cell bodies in the geniculate ganglion. In the rat this ganglion is a small elongated structure on the nerve (Hellekant, own observations). Its cell bodies do not extend centrally to where the facial root was cut in the expts. here. Further the cell bodies of the fibres are all in the CNS. Thus it seems that section at the point described here divides the fibres in the CT into two groups, a sensory one which still was connected with its cell bodies and a motor group which was disconnected from its cell bodies. According to an observation, first described by Waller (1852), this ought to cause degeneration of the motor fibres in the CT while the sensory fibres will remain. This seemed to be true, here the CT on the cut side contains about 25% fewer myelinated fibres and about 50% unmyelinated fibres than the normal side 18-20 days after section of the facial root (Hellekant, to be published). The sensory fibres seem vital both from morphological and physiological points of view because a good response to taste stimulation of the tongue is recorded from the peripheral part of the CT. No activity was observed in the motor fibres (Hellekant, own observations) which indicates that the efferent fibres had degenerated. In the normal rat nerve impulses can be recorded from the central part, as has been described earlier (Hellekant 1971 a, b, c, 1973 a, b, 1974). Thus it seems that the technique caused degeneration of the efferent fibres in the CT while the sensory remained unaffected. In spite of this, as has been described, electrical stimulation of the CT nerve caused significant dilatation of the blood vessels of the tongue. This suggests strongly that sensory impulses in the CT nerve cannot be used as an explanation for this dilatation. There are two other suggestions which must be dealt with before it can be concluded that there are vascular fibres to the tongue in the CT. One is that the vasodilatation observed in the rat, though it was recorded in the lingual artery, did not occur in the tongue or in any other structure. The main candidates seem to be glandular structures. However, the salivary glands, the submandibular and sublingual glands, could not be the cause, as they are not supplied by the lingual artery (Greene 1968, own observations). There are also glands in the posterior part of the tongue, von Ebner glands, but they are small and their hyperaemia was observed in the anterior part of the tongue. It might then be suggested that vasoactive substances were released from them and then carried to the anterior part of the tongue. This also seems highly improbable from both anatomical and physiological points of view because it would necessitate a quite different anatomy of the vascular system of the tongue from the one described (*cf* Hellekant 1972, 1976). If this was the case, then the veins from these tiny glands should drain into the arteries of the lingual artery. This is not the case. Therefore, it seems unlikely that the increased blood flow in the tongue to stimulation of the normal CT was caused by increased blood flow through these glands or an effect of activity in these structures. Finally it can be suggested that the vessels in the tongue of the rats whose facial nerve had already been cut were maximally dilated before their CT was stimulated. This might then explain the absence of an effect. However this explanation seems improbable for two reasons.



Fig. 4. The difference in temperature between the right and left side of the tongue has been recorded in a rat whose right facial root had been cut. The temperature was recorded in such a way that an upward deflection of the lower trace indicates an increase in temperature of the right side and a downward one an increase on the left side. The top trace shows from the left one stimulation of the left CT, two stimulations of the right CT and two stimulations of the left CT 40 Hz 10 μ A. The record shows that no change of tongue temperature could be elicited on the right side of the tongue.

Further the application of this method allowed us to compare the effect of cutting facial root and having it intact in the same animal. Fig. 4 shows an example of this. In the rat the right facial root had been cut while the left side was normal. A week after the cut the chorda tympani nerves on both sides were dissected. Their neural response to stimulation was recorded. It looked normal. Then the thermocouple was attached to the tongue and connected to the recorder in such a way that a downward deflection of the trace in Fig. 4 illustrates an increase of temperature on the left side, while an upward deflection shows an increase of temperature on the right side. The signal above indicates stimulation of the chorda tympani nerves 40 Hz, 10 μ A and 2 ms duration. The record in Fig. 4 shows that stimulation of the left chorda tympani nerve increased the temperature of the same side of the tongue, while stimulation of the right nerve—the side on which the facial root had been cut—caused no change of surface temperature. These experiments have been repeated in 5 animals with consistent results.

In conclusion, the results that have been described show that electrical stimulation of the peripheral part of the CT lowered the vascular resistance of the tongue, thereby confirming earlier observations by Hellekant (1970, 1972). Further the results indicate a hyperemia of the stimulated side of the tongue during the stimulation. Finally recordings from the surface of the tongue showed an increase in temperature on the stimulated side of the tongue. These effects disappeared after the facial nerve had been cut between the CNS and the geniculate ganglion a week to several months before the stimulation.

Discussion

It should perhaps be pointed out that this study is intended to be the first in a series which will deal with the vascular innervation of the tongue. The main purpose of this study is to rule out the explanation that the vasodilatation, which can be observed during stimulation of the CT, was the result of antidromic impulses in its sensory fibres, and to show that it is caused by nerve fibres to the vessels in the tongue. But before this conclusion can be drawn there are a number of factors which must be considered. The first is the method used to separate the sensory fibres from the motor fibres in the CT.

The CT is a branch of the facial nerve. It contains sensory fibres which mediate taste

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Firstly the perfusion flow through the tongues of these rats had not to be significantly higher than in the normal rats to maintain the blood pressure in the lingual artery. Second when a lethal dose of barbiturate was injected there was a large drop in the vascular resistance of the tongue in these rats. This indicates that these vessels were not dilated a maximum as the result of the denervation.

It seems from the above that there are specific nerve fibres to the vessels of the tongue which when stimulated dilate vessels. The only difficulty with regard to this conclusion seems to be the fact that recordings in some of these animals showed a decreased vascular resistance in the tongue to CT stimulation after the facial root was cut, though the decrease was small and only observed at the highest stimulus frequencies. The explanation for this is unknown. There are several possibilities. Since we observed that stimulation of the lingual proper nerve could have similar effects, this may indicate that antidromic impulses in sensory fibres may be able to elicit a slight effect provided the stimulation is strong enough. In an animal in which the maximum effect was observed (10%) some efferent nerve activity could be recorded from the central part of its CT which indicates that some regeneration may have occurred. Another explanation is that after section all efferent fibres had not completely degenerated in these rats. Finally we (Farbman and Hellekant, to be published) have compared the number of fibres in the part of the CT which runs along the tympanic membrane and the part immediately before the nerve joins the lingual proper nerve. Though on average there was no significant difference, data from some animals undoubtedly show that their peripheral CT receive a contribution of fibres whose origin has to be determined.

Any of the possibilities discussed above may be the cause for this slight vascular dilation which could be elicited after facial denervation in some of the rats. However it is felt that this does not make the conclusion that there are specific vasodilator fibres to the vessels of the tongue in the CT nerve invalid.

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Blood Pressure and Heart Rate Regulating Capacity of the Carotid Sinus during Changes in Blood Volume Distribution in Man

By

STURE BEVEGÅRD JAN CASTENFORS, LARS ERIK LINDBLAD and JUSSI TRANESÖ

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Abstract

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The influence of changes in blood volume distribution on the carotid baroreflex was studied in subjects. Blood volume distribution was changed by varying the pressure around the lower body up and below ambient, thereby varying the amount of blood pooled in this region and exerting a second influence on the central blood volume. The carotid arterial stretch receptors were stimulated by the pressure in an air-tight box enclosing the neck. To obtain a standardized carotid sinus sinus (SCS) the pressure in the box was varied sinusoidally between -10 and 40 mmHg with a frequency of 0.03 Hz. The effect on heart rate and blood pressure were assessed by harmonic analysis performed off-line on a digital computer. During lower body negative pressure of 40 mm (LBNP -40), i.e. during a procedure known to reduce the central blood volume, SCS induced an opposite effect on the blood pressure regulating capacity but not on the heart rate response. Expressing the blood pressure regulating capacity as peak-to-peak changes in systolic arterial pressure, the response during LBNP -40 mmHg was almost twice the control value. The opposite stimulus—lower body positive pressure—influenced the SCS-induced effects only slightly but on the average a minor reduction in blood pressure and heart rate regulating capacity was found compared with the control condition, though the difference did not reach significant levels.

The results support the hypothesis that changes in blood volume distribution modify the function of the carotid baroreflex, possibly via intrathoracic receptors sensitive to changes in central blood volume and/or central venous pressure.

Key words. Blood volume, carotid sinus, blood pressure, heart rate

Experiments in rats by Castenfors and Sjöstrand (1973) have demonstrated that hypervolemia decreases the blood pressure response to bilateral carotid occlusion while a moderate hypovolemia tends to increase the blood pressure response to this stimulus. Koike *et al.* (1975) demonstrated in anesthetized dogs an augmented gain of the carotid baroreceptor reflex after interruption of cardiopulmonary afferents. In a recent study Vatner *et al.* (1976) working with unanesthetized dogs, demonstrated that an acute volume load resulting in a

TABLE 1. Effect of LBNP -40 (n=6) and LBPP +40 (n=8) on intravascular pressures and heart rate. Mean values \pm S.E.

	Control	LBNP -40	Diff.	Sign. of diff.	Control	LBPP +40	Diff.	Sign. of diff.
heart rate	71.8	83.7	+11.8	p<0.01	69.3	70.3	+1.0	n.s.
heart rate ²	± 1.7	± 3.1	± 1.8		± 2.6	± 1.2	± 1.6	
mean arterial pressure	129.7	127.3	-2.3	n.s.	132.8	136.8	+4.0	n.s.
mean diastolic pressure	± 3.2	± 3.7	± 1.9		± 3.4	± 4.6	± 2.7	
central arterial pressure	93.5	93.8	+0.3	n.s.	94.6	99.0	+4.4	p<0.05
mean diastolic pressure	± 2.5	± 2.9	± 0.1		± 2.3	± 2.6	± 1.2	
mean systolic arterial pressure	80.2	85.0	+4.8	p<0.05	82.6	85.6	+3.0	n.s.
mean diastolic pressure	± 1.6	± 1.6	± 1.7		± 1.3	± 2.5	± 1.4	
central venous pressure	6.5	2.1	-4.3	p<0.01	7.5	8.8	+1.3	n.s.
mean diastolic pressure	± 1.0	± 1.3	± 0.5		± 0.7	± 1.0	± 0.6	

In two subjects LBNP -40 caused a progressive fall in blood pressure, resulting in an impending syncope which necessitated an abrupt termination of the stimulus. These two subjects have been excluded from the analysis. In the 6 subjects who maintained a relative circulatory steady state, LBNP induced a significant increase in heart rate and diastolic arterial pressure, while systolic arterial pressure and mean arterial pressure were not significantly changed. Central venous pressure decreased significantly (Table 1).

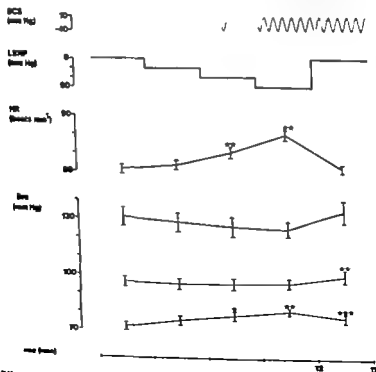


Fig. 1. Effect of gradually increasing LBNP stimulation on heart rate and blood pressure (mean values \pm S.E.). The asterisks give the level of significance for difference compared with control.

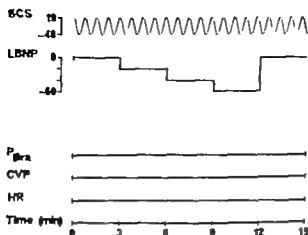


Fig. 2a.

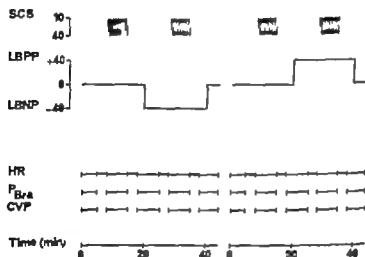


Fig. 2b.

Fig. 2a and b. Schematic presentation of applied stimuli and recorded variables.

of the sine wave that best fitted the resulting changes in heart rate and P_{Bra} (thus yielding information about the dynamic features of the baroreceptor system). Results concerning the central venous pressure were calculated manually. Details of the analysis and calculation of the baroreceptor system gain are given in the Appendix.

Statistical methods. The differences between data obtained during different blood volume distributions in each subject were analysed with Student's *t* test for paired data. Results are designated as follows: $p < 0.05$ x, $p < 0.01$ xx, $p < 0.001$ xxx. *P* values < 0.05 are considered significant.

General experimental design. A schematic presentation of the experimental set-up is given in Fig. 2a. The measurements were made with the subjects lying supine in a room with a temperature of 22°C . After the introduction of the catheters, the boxes surrounding the neck and the lower body were mounted. The subjects were then allowed to rest for about 10 min before starting the measurements. Two experimental designs were used as described in Fig. 2a and 2b respectively. The data on the dynamic effects of LBPP and LBPP per se refer to mean values of measurements before and after, as indicated in Fig. 2b.

Results

Effects of LBPP -40 and LBPP +40 on intracardiac pressures and heart rate

The circulatory effects of LBPP -40 and LBPP +40 respectively in these subjects are presented and discussed in detail in a separate article (Bevegård *et al.* 1977).

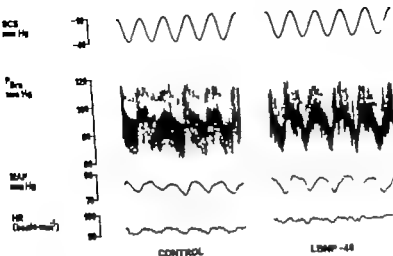


Fig. 5. Segment of an original recording during control condition and LBNP -40.

In none of the measured dynamic variables (*i.e.* peak-to-peak variation of heart rate, systolic arterial pressure, mean arterial pressure and diastolic arterial pressure) could a significant difference be demonstrated between the recordings during the two control tests in the same individual.

During LBNP -40. During LBNP -40 there were increases in peak-to-peak changes in systolic arterial pressure, mean arterial pressure and diastolic arterial pressure, though the rise reached significant levels only in the first two variables (Table II). In most cases the heart rate response was attenuated during LBNP -40, when several subjects displayed not only no rise but also fluctuation in heart rate (Fig. 5).

During LBPP +40. Comparing the findings during LBPP +40 with those during the preceding control period showed a slight but not significant decrease in the baroreceptor related peak-to-peak changes in blood pressure as well as heart rate (Table II).

During gradually increasing LBNP stimulation. Increasing the amount of blood pooled in the lower body by gradually decreasing the pressure surrounding this region, resulted in

TABLE II. Amplitude (peak-to-peak changes) in heart rate and blood pressure induced by SCS under control conditions and during LBNP -40 ($n=6$) and LBPP +40 ($n=8$). Mean values \pm S.E.

	Control	LBNP -40	Diff.	Sign. of diff.	Control	LBPP +40	Diff.	Sign. of diff.
Heart rate (beats min^{-1})	9.1 ± 1.7	7.0 ± 2.5	2.2 ± 1.7	n.s.	9.4 ± 2.2	8.7 ± 3.1	-0.7 ± 1.2	n.s.
Systolic arterial pressure (mm Hg)	6.0 ± 1.0	11.0 ± 1.2	5.0 ± 1.0	$p < 0.01$	8.8 ± 2.3	7.6 ± 1.2	-1.2 ± 1.3	n.s.
Mean arterial pressure (mm Hg)	6.8 ± 1.1	10.4 ± 1.4	3.6 ± 1.6	$p = 0.05$	8.5 ± 1.8	8.8 ± 1.3	-0.3 ± 0.9	n.s.
Diastolic arterial pressure (mm Hg)	6.8 ± 0.9	8.7 ± 1.8	1.9 ± 1.3	n.s.	7.5 ± 1.6	7.2 ± 1.2	-0.3 ± 1.1	n.s.

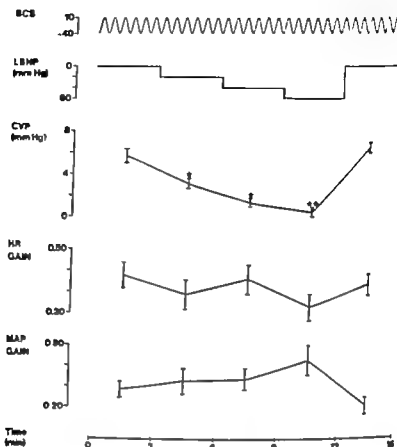


Fig. 4 Effect of gradually increasing LBNP stimulation on central venous pressure, heart rate (HR gain) and mean arterial pressure gain (MAP gain). Mean values \pm SEM. The asterisks denote the values of significance for difference compared with control. (Details of the calculation of the gain factors are in Appendix.)

LBNP +40 resulted in a slight but significant increase in mean arterial pressure, while the slight increases in systolic and diastolic arterial pressure were not statistically significant. Heart rate was essentially unchanged. (Table I) A significant increase in central venous pressure (+2.1 mmHg, $p < 0.05$) was found initially but central venous pressure gradually decreased towards the control value and during the period of SCS only a slight mean increase persisted, which was not statistically significant.

Circulatory effects of gradually increasing LBNP stimulation

Only data obtained during a simultaneous continuous SCS are presented. The hemodynamic changes induced were basically similar to those during LBNP -40. The magnitude of changes increased gradually with increasing LBNP stimulation. (Fig. 3 Fig. 4)

The sudden release of LBNP elicited a transient "overshoot" in central venous pressure amounting to 1.4 mmHg ($p < 0.05$) above the control value.

Carotid sinus stimulation—effects on blood pressure and heart rate

During control conditions. SCS in all cases caused blood pressure and heart rate to vary approximately sinusoidally with the same frequency as the input signal, as illustrated in

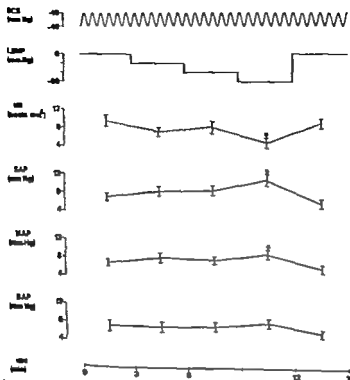


Fig. 7 Peak-to-peak changes in heart rate and blood pressure (Mean values \pm SEM) during gradually increasing LBNP stimulation. The asterisks give the level of significance for difference compared with control.

by a linear relation between dome and esophageal pressure. Kober *et al.* (1970) using an ultrasonic technique, demonstrated—for the pressure used in the present study—a linear relation between the increase in transmural pressure and the resulting increase in the radius of the carotid artery.

By using sinusoidally varying dome pressure, the effect could be calculated as the mean of several identical stimulations, thereby increasing the precision of the measurements. This stimulation method also has the obvious advantage of being suitable for harmonic analysis, using a computer as described in Appendix. As a consequence of the technique, however, the results of the analysis must be interpreted with caution the more the output signals deviate from the ideal sine wave (see Appendix).

Changing the pressure around the lower body is a well-documented method for changing the amount of blood pooled in the lower body thus influencing the central blood volume. The hemodynamic effects of LBNP and LBPP in these subjects are discussed in a separate article (Björklund *et al.* 1977).

The effects demonstrated on heart rate and blood pressure by RCS during control conditions in this study correspond well to earlier reports using neck section as baroreceptor stimulation (Björklund *et al.* 1966, Thron *et al.* 1967, Siegemann *et al.* 1970, Björklund *et al.* 1973 and Abboud *et al.* 1975).

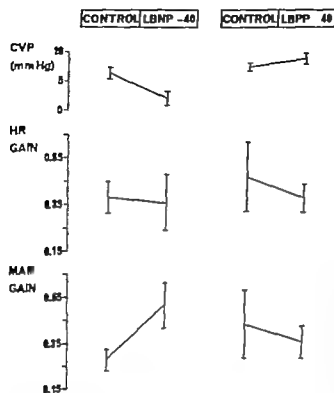


Fig. 6. Central venous pressure, gain (HR gain) and mean arterial pressure gain (MAP gain) during condition, LBPP -40 and LBPP +40 (Mean values \pm S.E.). Assess significance of deviation. (Data calculation of the gain factors in Appendix.)

a progressive decrease of central venous pressure and on the average, a slight increase in peak-to-peak changes of the blood pressure (Fig. 7), whereas the heart response became attenuated and distorted (Fig. 7 Appendix Fig. 8)

Baroreceptor system regulating capacity If changes in baroreceptor blood pressure regulating capacity are interpreted in terms of changes in the system gain factor (Appendix) average a slight though not significant decrease was found during LBPP (Fig. 6), in contrast to a significant increase during LBPP -40. During gradually increasing LBPP similar to a significant increase was found during LBPP -40 and LBPP -60 (Fig. 4).

In the period shortly after the release of the LBPP stimulus, however the blood pressure regulating capacity expressed as the mean arterial pressure gain factor showed a slight decrease compared with the control condition (Fig. 4)

If changes in heart rate regulating capacity induced by the carotid baroreceptor expressed in terms of system gain factors, no significant changes were found during LBPP -40 or LBPP +40 though a significant decrease was found during LBPP

Discussion Stimulation of the carotid arterial stretch receptors in man by applying atmospheric pressure around the neck was first used by Erling and Parry in 1967. A similar technique has been used since then by several authors (Bevegård and Shephard 1967, Thron *et al.* 1967, Wagner, Wachsbauer and Hilger 1968, Beiser *et al.* 1970, Kohn, Arndt 1970, Stegmann, Busert and Brock 1974, Abboud *et al.* 1975, Bjurstedt, Hamer and Tydén 1975).

The technique has been evaluated by for instance Thron *et al.* 1967 who showed that pressure applied was transmitted fairly well to the upper part of the esophagus, as in

agree that the blood pressure regulating capacity is increased during LBNP as evidenced by the significant increase in mean arterial pressure gain factor.

During LBNP the heart rate response became distorted, which means that the results concerning the heart rate regulating capacity during LBNP have to be interpreted with care (see Appendix). However it may at least be concluded that there is no indication of an increase in the baroreceptor mediated heart rate response.

During LBPP on the average, the blood pressure and heart rate response showed a slight, non-significant decrease. However LBPP induced only a transient increase in central venous pressure, and during the SCS period no significant increase in central venous pressure was found compared with control conditions. This may indicate that the increase in central blood volume was only minor during the SCS period and thus contributed to the finding of a small non-significant decrease in blood pressure and heart rate response to SCS. This is supported by the observation that after the sudden release of LBNP stimulus with continuous SCS, there was a significant decrease in the mean arterial pressure gain factor compared with control conditions. On release of the LBNP stimulus one would expect a rapid increase in central blood volume, possibly influencing receptors in the low pressure area (Brown *et al.* 1966), i.e. a hemodynamic situation similar to that caused by the onset of LBPP.

With the present data it is not possible to assess a conceivable modifying effect of the aortic baroreceptors on the effects of SCS under different experimental conditions. Data on the aortic baroreflex in man are very limited in the literature. However in anesthetized dogs the aortic baroreflex has been shown to respond mainly to changes in mean arterial pressure and only weakly to changes in pulse pressure (James and de Burgh Daly 1970). It has also been suggested that the aortic baroreflex is of importance mainly in a pressure range above the normal level (Donald and Edis 1971, Pfeffer, Clement and Shepherd 1972). If these results can be extrapolated to man, it is unlikely that under the conditions in this study in which mean arterial pressure deviated only slightly from control values, the effects of stimulating the carotid sinus would be influenced at all substantially by changes in the aortic baroreceptor activity.

A modifying effect of changes in arterial pressure, dp/dt , pulse pressure or heart rate on the carotid sinus cannot be excluded. If the findings in dogs are attributable to man, intrathoracic pressure (i.e. mean intrathoracic pressure) is the single most important determinant, whereas, in the physiological range, variations in pulse pressure and, particularly heart rate have relatively little reflex effect (Schmidt, Kumada and Sagawa 1972). In the present study during SCS the mean blood pressure during LBNP - 60 deviates from the control level by only 1 mmHg. It is therefore unlikely that changes in mean arterial pressure account for the significant increase that was demonstrated in baroreceptor sensitivity during LBNP.

Evidence that pooling of blood in the lower body decreases the filling pressure of the heart, i.e. the transmural pressure, has been presented by Murray *et al.* (1968). They demonstrated that even LBNP - 60 caused only minor and inconsistent decreases in intrathoracic pressure as assessed from the esophageal pressure (range 0-2.2 mmHg), whereas central venous pressure showed a consistent decrease of the order of 7 mmHg.

This suggests that LBNP ought to provide an adequate stimulus for vascular receptors

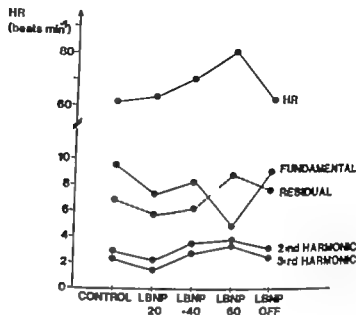


Fig. 8a

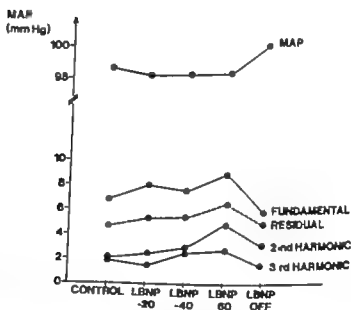


Fig. 8b

Fig. 8. Amplitudes (peak-to-peak values) of fundamental second and third harmonic sinusoidal components during increasing LBNP stimulation. A Heart rate. B Mean arterial pressure. To maintain relation between the fundamental and residual, the latter is expressed in effective value. As a reference mean values of mean arterial pressure and heart rate are also given (top of diagrams).

The hemodynamic changes induced by LBNP -40 resulted in a significant increase in the baroreceptor mediated blood pressure response and during gradually increasing LBNP stimulation there was a progressive increase in the blood pressure response. Although the carotid sinus blood pressure buffering system is not an ideally linear function, the result

qual quality

the fluctuations of heart rate and systolic, mean and diastolic pressures in response to SCS were, as expected, basically sine-wave shaped. However, harmonics and noise were included. In Fig. 8 the decompose between the fundamental sine wave, the first two harmonics and the residual are shown for heart rate and mean arterial pressure during increasing levels of LBNP. Results are based on the closed-circuit system. Results of the same magnitude range were obtained for LBNP -40 and LBNP +40.

As demonstrated, the residuals and the fundamentals are of the same magnitude. The relatively large residuals are interpreted as partly consequence of the normal physiological fluctuations in heart rate and LAP. For example, the normal sinus arrhythmia in resting young men has a magnitude of approx. 6-7 beats/min (Schönberg 1937) and the same frequency as respiration, i.e. 10-15 periods/min. The frequency corresponds to the 5th-8th harmonics in this study thus augmenting the residual. The second and third harmonics are only 5 times smaller than the fundamentals, indicating the non-linearity of the baroreceptor system.

The authors wish to thank Hans Karlsson, Department of Medical Engineering, Linköping University, for valuable advice on the harmonic analysis.

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on the venous side that are sensitive to changes in transmural pressure. Thus the findings of this study might be interpreted as supporting studies mentioned earlier by Castenfors *et al.* (1973) Vatner *et al.* (1975) and Koike *et al.* (1975), suggesting that impulses from intrathoracic receptors, possibly in the low pressure area, modify the function of the carotid sinus system.

Appendix

Signal analysis

(The electrocardiogram (ECG), the brachial artery pressure (P_{Bra}) and the aortic pressure were recorded on an analog FM tape recorder (Tandberg, model 100) for subsequent off-line computer (Nico) analysis. Heart rate (HR) as well as systolic, diastolic and mean pressures were calculated beat-to-beat (Nygård *et al.* 1976). To obtain steady state values, computer analysis was always started at least 1 min after a change in external conditions.

Time equidistant signal values are required as input of the final harmonic analysis described below. Therefore, HR and P_{Bra} were computed at 1 s intervals assuming linear changes between consecutive beat-to-beat values.

According to control theory harmonic analysis (Elgerd 1967) of a control system can be used to describe system features if the input and output signals are known. With a sine wave (amplitude A , frequency

$$x(t) = A \sin 2\pi ft + C_1$$

as input, the output $y(t)$ will generally be a sum of sine waves

$$y(t) = \sum_{n=1}^{\infty} B_n \sin(n 2\pi ft + \phi_n) + C_2$$

where B_n and ϕ_n represent the amplitude and phase lag of the n th harmonic wave with the frequency $n f$. C_1 and C_2 are the offset (DC) values of the input and output signals respectively. Note that the fundamental output wave ($n=1$) has the same frequency as the input.

For a linear system, all harmonics equal zero. If the fundamental is large compared with the harmonics the non-linear system could be approximated as linear (within certain limits).

A computer program for harmonic analysis (Broeman and Wigertz 1967) was used in this study. The computer calculated the sine wave that best described the recorded fluctuations in the output signal, i.e. heart rate and blood pressures. In addition, the first two output harmonics were calculated. Information loss by using the fundamental and its first two harmonics to describe the results is given as "residual" which is presented here as the effective value of remaining harmonics and spontaneous fluctuations ("noise").

A total of four sine wave periods were A/D-converted. Due to the relatively high "noise" level of the output signal (Appendix Fig. 8) the three initial and the three final periods were analysed independently. The results presented here are based on the three periods having the smallest residual, thus giving the best description of the output signal.

Gain factor

To verify that the demonstrated effects depend on a real change in buffering capacity control theory can be applied by regarding the reflex as a closed loop system which is disturbed by the pressure surrounding the sinus wall (Stegemann *et al.* 1974).

Assuming that intrasinus blood pressure equals the pressure measured in the brachial artery and that subatmospheric pressure applied around the neck is almost completely transmitted to the sinus wall (as quoted in a review by Wolthuis, Bergman and Nicogossian 1974 and Kober *et al.* (1970)) the effective transmural pressure (TMP) was calculated as the difference between mean arterial pressure and diastolic pressure.

In the computerized harmonic analysis, the gain factor (defined as the amplitude of the output sine wave divided by the amplitude of the input sine wave, i.e. B_1/A according to eq. 1) as a function of heart rate and MAP was first calculated regarding the diastolic pressure as input signal (closed loop system). By transferring the results with regard to control theory (Elgerd 1967), the gain factors were obtained regarding the TMP as system input (open loop gain).

Changes in ATP and Cyclic Nucleotide Levels during Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue *in situ*¹

By

BERTIL B. FREDHOLM, ERIK BELFRAAGE and ELEONORA BLANCHICE

Received 30 August 1976

Abstract

FREDHOLM, B. B., E. BELFRAAGE and E. BLANCHICE. *Changes in ATP and cyclic nucleotide levels in canine subcutaneous adipose tissue during sympathetic nerve stimulation.* Acta physiol. scand. 1977 99 313-322.

Subcutaneous adipose tissue *in situ* in fed, female dogs was isolated. Biopsies of the tissue (30-150 mg) were taken and rapidly frozen in liquid nitrogen before, during and after nerve stimulation (3-4 Hz). In unstimulated adipose tissue the levels of ATP were 74 ± 7 nmol/g, of cyclic AMP 90 ± 12 pmol/g and of cyclic GMP 18 ± 3 pmol/g (mean \pm S.E.). During sympathetic nerve stimulation the levels of ATP and cyclic GMP fell by 30 and 50% respectively ($p < 0.01$), while the cyclic AMP content increased by 4% ($p < 0.05$). After nerve stimulation there was a marked increase in glycerol release, and the levels of all three nucleotides returned to control. The fall in ATP during nerve stimulation was essentially prevented by prior adrenergic α -receptor blockade. It is concluded that 1) sympathetic nerve stimulation induces a rapid, reversible fall in tissue ATP content, which may be related to hypoxia secondary to the vasoconstriction, and 2) lipolytic responses to sympathetic nerve stimulation *in vivo* are preceded by small increases in the tissue cyclic AMP level, and a 3-fold increase in the cyclic AMP/cyclic GMP ratio.

Stimulation of the sympathetic nerves to canine subcutaneous adipose tissue has vascular as well as metabolic effects. For example, there is vasoconstriction due to stimulation of vascular adrenergic α -receptors (Ngai *et al.* 1966), and an increased rate of lipolysis, glycolysis and oxygen consumption due to stimulation of adrenergic β -receptors (Fredholm and Rosell 1968, Fredholm and Karlsson 1970).

There is good evidence that lipolysis in isolated fat cells is regulated by cyclic AMP (cf. Fain 1973), but so far there is only circumstantial evidence that cyclic AMP regulates lipolysis in canine subcutaneous adipose tissue *in situ* (Fredholm 1974). In several mammalian cell systems evidence has been presented that cyclic AMP and cyclic GMP may mediate

¹The following abbreviations were used: ATP-adenosine 5'-triphosphate; ADP-adenosine 5'-diphosphate; AMP-adenosine 5'-monophosphate; cyclic AMP-adenosine 3',5'-cyclic monophosphate; cyclic GMP-guanosine 3',5'-cyclic monophosphate; PCA-perchloric acid; EDTA-ethylenediamine tetraacetic acid; FFA-free (unesterified) fatty acids.

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Changes in ATP and Cyclic Nucleotide Levels during Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue *in situ*¹

By

BERTIL B. FREDHOLM, ERIC BELFRAGE and ELFONORA BLANCHKE

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Abstract

FREDHOLM, B. B., E. BELFRAGE and E. BLANCHKE. Changes in ATP and cyclic nucleotide levels in canine subcutaneous adipose tissue during sympathetic nerve stimulation. *Acta physiol. scand.* 1977 99 313-322.

Subcutaneous adipose tissue in fed, female dogs was isolated. Biopsies of the tissue (30-150 mg) were taken and rapidly frozen in liquid nitrogen before, during and after nerve stimulation (3-4 Hz) in minimised adipose tissue. The levels of ATP were 74 ± 7 nmol/g, of cyclic AMP 90 ± 12 pmol/g and of cyclic GMP 18 ± 3 pmol/g (mean \pm S.E.). During sympathetic nerve stimulation the levels of ATP and cyclic GMP fell by 30 and 50% respectively ($p < 0.01$), while the cyclic AMP content increased by 4- to 6-fold. After nerve stimulation there was a marked increase in glycerol release, and the levels of β these nucleotides returned to control. The fall in ATP during nerve stimulation was completely blocked by prior adrenergic α -receptor blockade. It is concluded that 1) sympathetic nerve stimulation causes a rapid, reversible fall in tissue ATP content, which may be related to hypoxia secondary to the vasoconstriction, and 2) lipolytic responses to sympathetic nerve stimulation *in situ* are preceded by small changes in the tissue cyclic AMP level, and a 3-fold increase in the cyclic AMP/cyclic GMP ratio.

Stimulation of the sympathetic nerves to canine subcutaneous adipose tissue has vascular as well as metabolic effects. For example, there is vasoconstriction due to stimulation of vascular adrenergic α -receptors (Ngai *et al.* 1966), and an increased rate of lipolysis, glycerololysis and oxygen consumption due to stimulation of adrenergic β -receptors (Fredholm and Rosell 1968, Fredholm and Karlsson 1970).

There is good evidence that lipolysis in isolated fat cells is regulated by cyclic AMP (cf. Fahn 1973), but so far there is only circumstantial evidence that cyclic AMP regulates lipolysis in canine subcutaneous adipose tissue *in situ* (Fredholm 1974). In several mammalian cell systems evidence has been presented that cyclic AMP and cyclic GMP may mediate

¹The following abbreviations were used: ATP=adenosine 5'-triphosphate; ADP=adenosine 5'-diphosphate; AMP=adenosine 5'-monophosphate; cyclic AMP=adenosine 3',5'-cyclic monophosphate; cyclic GMP=guanosine 3',5'-cyclic monophosphate; PCA=perchloric acid; EDTA=ethylenediamine tetraacetic acid; FFA=free (unesterified) fatty acids.

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Cyclic AMP was measured on 50 μ l aliquots of plasma or neutralized tissue extracts by competitive binding assay (Brown *et al.* 1972). The binding protein was prepared from bovine adrenal cortex. 3 H-cyclic adenosine 3',5'-monophosphate (27.5 Ci/mMol) was obtained from the Radiochemical Centre, Amersham, England and unlabelled cyclic AMP from Boehringer Mannheim, West-Germany. Recovery of 3 H-cyclic AMP added at the homogenization step was essentially complete. Purification of the samples was not necessary (Faruqi *et al.* 1976).

Cyclic GMP was measured by radioimmunoassay according to Secler *et al.* (1973) as modified by Harper & Brooker (1975). 2'-O-acetyl-cyclic guanosine 3',5'-monophosphate (Sigma, St. Louis, USA) was coupled to keyhole limpet haemocyanin (Calbiochem, USA) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The antigen was injected subcutaneously into rabbits every 3-4 weeks to generate the antisera. The antisera were used at final dilution of 1:20 000 to 1:50 000. Unlabelled cyclic GMP (Boehringer, Mannheim), samples or blanks were treated with acetic anhydride in triethylamine to silylate the cyclic nucleotide. Incubation for 3 h at 4°C using 125 I- γ -cytosyl-succinyl cyclic GMP (Schwartz, Orangeburg, USA) as the labelled antigen. Free and bound cyclic GMP was separated by the addition of 4 volumes of 60% saturated ammonium sulfate. Amounts of cyclic GMP between 0.1 and 5 pMol were determined with precision of 8 per cent. Cyclic AMP, AMP, ADP, ATP, GDP, GTP did not interfere with the assay at 1 000 times higher concentration. Purification of the samples on short columns Dowex 50X-4 did not alter results.

ATP was determined by the luciferase-luciferin method essentially as described by Stanley and Wilson (1969). The enzymes were extracted from desiccated tad and luciferase (Sigma) with 1 M sodiumsuccinate buffer pH 7.4 one day in advance to maximize blank readings. The assay was done at room temperature in 1.9 ml final volume of 30 mM sodiumsuccinate, 11 mM magnesium chloride and 0.2 ml of diluted tissue extract. The reaction was started by the addition of luciferin extract. On the Packard 2048 liquid scintillation spectrometer was started to count the emitted photons for period of 6 s. The optimal setting as found to be out of coincidence, 100% amplification and 40-60 times setting of the discriminator. Sample readings (in duplicate) were compared with standards (1:50) and it has been reported that ATP coprecipitates with potassium perchlorate (Werner *et al.* 1974). The low ATP may be appreciable at low ATP levels and at high concentrations of perchloric acid. Under our conditions the recovery of 3 H-ATP in the supernatant after precipitation was 85-98 per cent. Corrections for the loss were not carried out.

Statistics. Results are expressed as mean \pm S.E. and hypotheses were tested by the Mann-Whitney test or the Student's *t*-test.

Results and discussion

Adipose levels under basal conditions

The level of ATP found in biopsies from canine subcutaneous adipose tissue, 73.6 ± 6.7 nmol/g w.w. (n = 21) agrees with that reported for other species (30-110 nmol/g w.w., Denison *et al.* 1966, Hepp *et al.* 1968, Böhler and Jeanrenaud 1970, Hanson and Ballard 1970, Angel *et al.* 1971, Knight and Illife 1973, Fredholm and Froese 1974). This suggests that ATP levels in adipose tissue are quite similar in different species and that the present technique has been adequate.

Similarly the value for cyclic AMP content in canine subcutaneous adipose tissue, 90 ± 12.6 pmol/g w.w. (n = 28) found in the present study falls within the range reported for rat and human adipose tissue (37-180 pmol/g w.w. Butcher *et al.* 1965, Fassina *et al.* 1972 a, Knight and Illife 1973, Arner and Östman 1975). There is little reported information about the cyclic GMP content in adipose tissue. We found a cyclic GMP level of 17.7 ± 2.9 pmol/g w.w. (n = 8) which is similar to the values recently reported for rat parametrial fat cells (7.18 pmol/g w.w.) by Falk and Butcher (1976). Illiano *et al.* found between 6 and 30 pmol cyclic GMP per million fat cells. The corresponding value for cyclic AMP reported by most authors is around 40 pmol/ 10^6 cells (e.g. Fredholm and Hjemdahl 1976). This corresponds

opposing effects and that the magnitude of the response may be determined by the ratio between cyclic AMP and cyclic GMP in the tissue (Goldberg *et al.* 1975). A report by Hase *et al.* (1973) on the effect of insulin and acetylcholine in isolated fat cells suggests that the lipolytic response may also be governed by the cyclic AMP/cyclic GMP concentration ratio.

The relationship between ATP and lipolysis seems to be complex. ATP is necessary for the synthesis of cyclic AMP and also for the activation of hormone sensitive lipase by protein kinase (Huttunen *et al.* 1970). It has been found that inhibitors of glycolysis or oxidative phosphorylation depress fat cell ATP levels and inhibit lipolysis before and after the step of cyclic AMP formation (Hollenberger and Patten 1970, Fassina *et al.* 1972a, 1972b). Conversely the ATP levels in adipose tissue are depressed by lipolytic hormones (Hepp *et al.* 1968, Bihler and Jeanrenaud 1970, Angel, Desai and Halprin 1971). The fall in ATP under *in vitro* conditions appeared to be strongly dependent on the accumulation of intracellular fatty acids (Angel *et al.* 1971). Under *in vivo* conditions other factors may also play a role in the regulation of adipose tissue ATP levels. Thus, when vasoconstriction is induced in adipose tissue there is a pronounced fall in the tissue oxygen tension down to levels where the oxygen supply may limit ATP synthesis (Fredholm *et al.* 1976).

In the present study we have examined the effect of sympathetic nerve stimulation on adipose tissue cyclic nucleotide and ATP levels under *in vivo* conditions. We report that sympathetic nerve stimulation induces a reversible fall in the ATP level and an increase in the cyclic AMP/cyclic GMP concentration ratio. Some possible explanations and implications of these findings are briefly discussed.

Materials and methods

Animals. 9 fed female mongrel dogs weighing 9–15 kg were used. Anaesthesia was induced by sodium pentobarbital (Nembutal, Abbot, 25–30 mg/kg i.v.) and was supplemented whenever necessary. Heparex 1 000–2 500 U/kg was given 30–60 ml before the experiment to prevent clotting of blood. The animals were tracheotomized and artificially ventilated.

Experimental. Subcutaneous adipose tissue was isolated (Rosell 1966) and autoperfused from femoral artery. An arterial drop counter was used to monitor blood flow. The venous effluent was returned to the animal via the femoral vein. Adipose tissue blood flow and systemic arterial blood pressure (Statham P23 AC) were recorded on a Grass Polygraph. Samples (1–4 ml) of arterial and venous blood were taken at times containing 20–50 μ l 0.5 M sodium citrate-EDTA. These tubes were kept on ice until centrifuged. Aliquots of plasma were frozen for subsequent analysis of cyclic AMP, ATP or radioactivity or were deproteinized for the assay of glycerol (see below).

The cut nerve to the adipose tissue preparation was placed on bipolar platinum (or silver) electrodes protected from drying by Plastibase (Squibb), and stimulated at the rate of 3–4 Hz with pulses of maximal intensity (12–15 V) and duration (2 ms) delivered by a Grass 7B stimulator.

Tissue handling. Biopsies of 30–150 mg of the perfused adipose tissue were rapidly cut out and frozen in liquid nitrogen. The time between the start of taking the biopsy until freezing did not exceed 2 s. The biopsies were kept at -80°C or in liquid nitrogen until they were crushed between airtight blocks precooled by liquid nitrogen. The powdered material was quickly poured in tarred homogenizer tubes containing 1 ml 0.4 N PCA, weighed and homogenized. After centrifugation an aliquot of the clear layer between the precipitate and fat cake was taken and neutralized with 4 N KOH and sufficient 1 M Tris base to make the final concentration 0.05 M. The neutralized extract was stored at -20°C until assayed.

Assays. Glycerol was measured enzymatically as described by Laurell and Tibbling (1966). Results are expressed as μ mol glycerol released per minute and 100 g tissue.

TABLE II Effect of sympathetic nerve stimulation on adipose tissue cyclic nucleotide levels. Mean \pm S.E. Number of determinations within parentheses

	Cyclic nucleotide level in % of control			
	During stim.		After stim.	
	2-4 min ^a	7-12 min ^a	2-3 min ^b	7-12 min ^b
lc AMP	114 \pm 18 (9)	152 \pm 31 (12) <i>p</i> < 0.05	123 \pm 17 (9)	95 \pm 16 (9)
lc GMP	99 \pm 35 (4)	44 \pm 15 (6) <i>p</i> = 0.01	146 \pm 33 (7)	141 \pm 37 (10)

stim cyclic AMP was 90.0 ± 12.0 (28) pmol/g w.w. and control cyclic GMP was 17.7 ± 2.9 (8) pmol/g w.w. After α -blockade cyclic AMP at 130 ± 33 (8) pmol/g cyclic GMP 10.3 (7) pmol/g; after β -blockade values were 82 ± 13 (4) and 10.0 (2).

^a Data is from after the start of stimulation.

^b Data is from after the end of stimulation.

Stimulation difference for cyclic AMP found during resting condition was enhanced, rather than diminished or reversed, during nerve stimulation (average venous cyclic AMP 11.4 nM). After stimulation the venous cyclic AMP levels returned to control. The decreased venous cyclic AMP during nerve stimulation probably results from the decreased blood flow and consequently increased time of exposure of cyclic AMP to phosphodiesterase in plasma and in the formed elements. Even so, our results suggest that adipose tissue does not contribute significantly to the increase in plasma cyclic AMP levels seen during catecholamine stimulation (Ball et al. 1971). In recent study Issekutz (1975) similarly concluded that there is no relationship between plasma cyclic AMP turnover and FFA mobilization from adipose tissue. Conversely in situations of high sympathoadrenal tone, such as hemorrhage, there may be large increase in plasma cyclic AMP levels with no increase in the plasma level of glycerol or FFA (Parnes et al. 1976).

The results presented in Table II further show a significant fall in tissue cyclic GMP levels during nerve stimulation. Fain and Butcher (1976) recently reported that noradrenaline increases cyclic GMP levels in rat fat cells under their basal conditions. However when the cells were exposed to insulin, fatty acids or a calcium ionophore, which by themselves raise cyclic GMP noradrenaline sometimes produced a decrease in cyclic GMP levels. Adipose tissue *in situ* is exposed to insulin and furthermore the level of FFA seems to be high (Fredholm 1970, Kashyap et al. 1975). Therefore the difference between our results and those of Fain and Butcher may be due, at least in part, to differences in the basal conditions. Our finding of markedly elevated cyclic AMP/cyclic GMP ratio in parallel with an enhanced lipolysis (Table I) may support the idea that cyclic GMP is of importance for lipolysis. However, Fain and Butcher (1976) failed to demonstrate any relationship between cyclic GMP and lipolysis in rat fat cells. Furthermore Fredholm (1974) found that dibutyryl cyclic GMP had minimal effects on basal or stimulated lipolysis in the isolated perfused canine epididymal adipose tissue. Thus the physiological significance of the present finding is obscure.

TABLE I Effect of nerve stimulation on lipolysis (glycerol release), vasoconstriction and on the cyclic AMP/cyclic GMP ratio

	Glycerol release $\mu\text{mol min}^{-1} 100 \text{ g}^{-1}$	Vascular resistance PRU_{100}	Cyclic AMP Cyclic GMP
Cont. of	0.24 ± 0.10	21 ± 4	5.1
During stim. 2-5 min.	0.19 ± 0.07	95 ± 26	6.5
During stim. 9-12 min.	0.94 ± 0.24	60 ± 10	17.6
After stim. 2-3 min.	5.69 ± 1.64	14 ± 1	4.3
After stim. 4-6 min.	4.05 ± 1.02	12 ± 1	—
After stim. 7-11 min.	1.87 ± 0.33	14 ± 1	3.4

to a cyclic AMP/cyclic GMP ratio of between 1.5 and 10. We found a ratio between 3 and 8 (Table I).

Changes in cyclic nucleotide levels during nerve stimulation

The changes in cyclic nucleotide levels during nerve stimulation are summarized in Table I. The increase in cyclic AMP induced by nerve stimulation is small—the average increase is 50% over basal. In view of the very large increases in cyclic AMP reported following catecholamine stimulation *in vitro* (e.g. Butcher *et al.* 1965; Knight and Little 1973; Fredholm and Hjemdahl 1976) it may be argued that these *in vivo* changes are artefactually small. As shown in Table I the changes in glycerol release and in vascular resistance are large suggesting that the limited increase in cyclic AMP cannot be due to a failure of the electrical stimulation. On the other hand, it is possible that lipolysis is induced from a different population of cells (e.g. cells situated deep within the tissue) than those sampled by the biopsies. It should be pointed out that the lipolytic rates observed in these expts. are close to the maximal lipolytic rates obtained in this preparation of adipose tissue by any procedure including *i.a.* infusion of isoprenaline (Ballard *et al.* 1971) or theophylline (Fredholm 1974) in massive doses. Therefore a selective activation of only a small number of cells seems unlikely but cannot be excluded. It is of interest that the *i.v.* infusion of NA at a rate of 0.5 $\mu\text{g/kg/min}$, which produces plasma levels of NA close to $2 \cdot 10^{-8}$ M produces a small increase in lipolysis in the canine subcutaneous adipose tissue (Hjemdahl and Fredholm 1976). In isolated rat fat cells this concentration of NA produces an approximately similar increase in lipolysis, while alteration in cyclic AMP are undetectable (Fredholm and Hjemdahl 1976).

The possibility must thus be borne in mind that maximal or close to maximal lipolytic responses in the intact canine s.c. adipose tissue are associated with small, almost undetectable alterations in the absolute level of cyclic AMP.

In some tissues, such as the liver, catecholamine stimulation is followed by a very moderate change in total tissue level of cyclic AMP while there is a dramatic increase in the release of cyclic AMP to the extracellular medium (Exton *et al.* 1972). In order to investigate whether this is true also for adipose tissue, arterial and venous samples were analyzed for cyclic AMP. The arterial concentration averages 19.8 nM (range 14.1–23.6, $n=8$), while the venous was 18.2 nM (range 14.8–23.9 nM, $n=8$). This slight positive arteriovenous con-

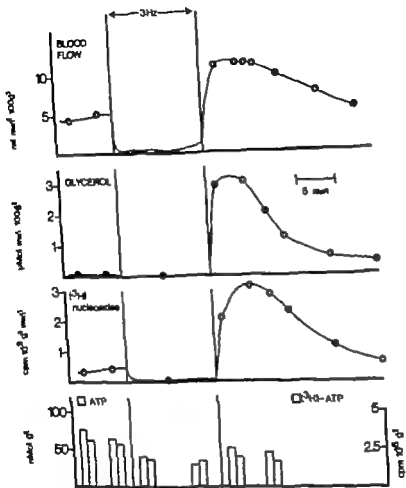


Fig. 2. Effect of sympathetic nerve stimulation (3 Hz, 11 V, 2 sec for 12 min) in canine subcutaneous adipose tissue (Q 2) labelled with ^3H -adenine 90 min previously. For further explanation see text.

These values correspond to a "release" by adipose tissue of some 30 pmol ATP/min/g. The exact source of this ATP is not completely known, however. Thus, release from erythrocytes and platelets may be important (Forrester and Lind 1969). Furthermore, there was no change in the veno-arterial concentration difference for ATP by nerve stimulation in these experiments.

In 3 expts. the tissue adenine nucleotide stores were labelled by an infusion of ^3H -adenine. As seen in Fig. 1 most of the tritium is present in nucleotides, of which ATP predominates. The results obtained in one of these expts. are illustrated in Fig. 2. The stimulation produced a marked vasoconstriction, followed by a period of hyperemia, during which glycerol release was substantially increased. During nerve stimulation there was a parallel fall in non-radioactive and radioactive ATP. In agreement with earlier results (Frédholm 1976) nerve

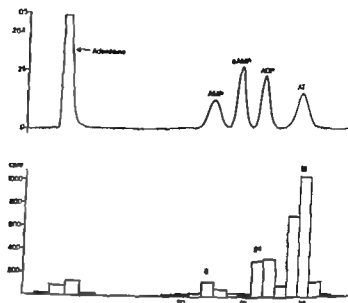


Fig. 1 Distribution of radioactivity in canine subcutaneous tissue labelled with ^3H -adenosine (100 min previously). Biopsies were cut out, frozen in liquid nitrogen, homogenized in 0.4 M perchloric acid. Protein free fat infranant was neutralized with 4 M KOH and 1 M Trisbase. 0.6 μmol ADP, 0.6 μmol Adenosine and 0.15 μmol cAMP were added as carrier and for identification of radioactive A. A total volume of 1.6 ml sample was applied on top of a 0.9 \times 22 cm column of AGI 2-200 chloride form. A gradient of HCl in water was used for elution. Absorbance was monitored on a model absorbance monitor at 254 nm with a flowcell having a light path of 0.5 cm. Fraction 28

Changes in ATP levels during nerve stimulation

During nerve stimulation there was a rapid fall in tissue ATP content, which lasted for minutes following stimulation but later the ATP content returned to control (Table III). In some expts. the ATP levels in plasma from arterial and venous blood were measured. We found approximately 0.6 ($n=4$) μM ATP in arterial blood and 0.9 μM ($n=8$) in venous blood, i.e. levels similar to those found by Forrester and Lind (1969) in the human fat.

TABLE III ATP levels in adipose tissue before, during and after nerve stimulation. Mean \pm S.E. within parentheses represent number of expts.

	ATP level % of control			
	During stim.		After stim.	
	2-4 min ^a	7-12 min ^a	2-3 min ^b	7-12 min ^b
No drug	78 \pm 12 (8) $p < 0.01$ ^b	71 \pm 6 (11) $p < 0.01$	73 \pm 8 (9) $p < 0.01$	97 \pm 16 (7)
α -Adrenoceptor block de ^a	100 \pm 12 (6)		116 \pm 17 (5)	

Control ATP levels were 73.6 \pm 6.7 (21) with no drug treatment and 53.1 \pm 10.9 (7) nmol/g ww α -adrenoceptor blockade.

Hydargine 250-300 μg i.a. Phentolamine 700 μg i.a.

^a Student's *t* test.

^b Refers to time after the start of stimulation.

^c Refers to time after the end of stimulation.

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stimulation was associated with a marked increase in the release of radioactive (acid nucleoside) material. Based on the specific activity of the tissue ATP (4.3 ± 10^6 cpm/nmol) it can be calculated that totally about 0.8 nmol purine material was released per g tissue. This corresponds to only 1% of the total ATP content. Thus release of ATP per se or in a form of nucleosides cannot be held responsible for the fall in ATP during nerve stimulation.

Furthermore, the fall in ATP cannot be accounted for by a rise in cyclic AMP since it was three orders of magnitude less. Thus, the most reasonable explanation is that nerve stimulation induces a shift in the nucleotide pool from ATP to less energy-rich compounds. Support for this contention is afforded by preliminary findings that ^3H -nucleotides, separated as described in Fig. 1 in biopsies taken immediately following nerve stimulation, shifted towards ADP and AMP.

As noted in the Introduction a fall in ATP levels has been found also in fat cells and adipose tissue *in vitro*. This seems to be due, at least in part, to the accumulation of intracellular fatty acids, which may inhibit ATP synthesis (Angel *et al.* 1971). The fall seen in the present study appears to be more rapid than that observed *in vitro*. It is therefore possible that it has a different explanation. It was recently found that adrenergic stimulation causes a rapid fall in tissue oxygen tension (Fredholm *et al.* 1976). This seems to be due to the combined effect of a decreased gross blood flow, a decreased number of open capillaries and a stimulated metabolism, or in other words due to both α -adrenoceptor actions on the vasculature and β -adrenoceptor actions on metabolism. We therefore carried out some experiments in tissue treated with α -adrenoceptor blocking agents, which eliminate the vasoconstrictor effect of nerve stimulation. As seen in Table III the effect on ATP levels were also abolished. This supports the idea that the shift in the adenine nucleotide pool seen as a fall in ATP in adipose tissue may be due to local hypoxia secondary to decreased blood perfusion and a decreased number of open capillaries.

Under the present experimental conditions the fall in ATP was reversible. However, if hypoxia is prolonged, e.g. in hemorrhagic shock, there is an irreversible fall in ATP levels (Fredholm and Fronek 1974). There are several possible explanations for this irreversibility. First, there may be irreversible damage to the ATP synthesizing machinery in the cells, for example to the mitochondria. Secondly, the release of purine compounds may, if produced sufficiently, lead to a depletion of substrate for ATP synthesis. Since the ATP depletion in cells in certain target organs during shock and other traumatic conditions probably accounts for an important part of irreversible tissue damage, it is of considerable therapeutic interest to find out the mechanisms involved. The present results suggest that adipose tissue may be a useful organ to pursue such studies in.

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Renal Na-K ATPase Activity during Saline Infusion in the Rabbit

By

OLE M. SEJVED

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Abstract

EDVARD O. M. *Renal Na-K ATPase activity during saline infusion in the rabbit* Acta
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During saline infusion, sodium reabsorption (R_{Na}) in the diluting segment (thick ascending limb of Henle's loop) increases steadily. The mechanism for this higher pumping rate of active medullary Na-K ATPase is unknown. Following left-sided nephrectomy immediate infusion of hypertonic saline increased R_{Na} in the remaining sole right kidney by 23 ± 14 ($p < 0.05$). Na-K-ATPase activity in outer medulla was raised by $(\Delta) 23 \pm 4$ above the left kidney ($p < 0.05$), whereas cortical activity was unchanged. The mechanism for this increase in Na-K-ATPase activity was explored. The catalytic rate per enzyme did not differ in the two kidneys and amounted $5.940 \text{ } \mu\text{mol}^{-1}$. The increase was therefore due to higher tissue concentration of active enzyme. The response was fully developed during continuous infusion within 20 min, and of equal magnitude. Ischaemic protein synthesis had been inhibited by cycloheximide ($\Delta -23 \pm 7$) or abolished by unilateral nephrectomy 6 days earlier combined with saline infusion for 2 h ($\Delta -34 \pm 10$). Thus, during hypertonic saline infusion, the increased R_{Na} in the outer medulla was partly accounted for by the activation of latent Na-K-ATPase. High delivery of sodium to the diluting segment for more than 2 h during hypertrophy caused no further activity change.

Key words: Active sodium reabsorption, cycloheximide, enzyme activation, ion transport, kidney medulla, loop of Henle, renal hypertrophy

Saline infusion increases both the sodium reabsorption in the thick ascending limb of Henle's loop (diluting segment) (Rector *et al.* 1964, Schnermann 1968, Kunau *et al.* 1974) and the metabolic rate of the outer medulla (Kilb *et al.* 1971). Several reports suggest that sodium reabsorption is carried out in this segment by Na-K ATPase (Katz and Epstein 1967, Nozhey and Nelson 1970), located at the peritubular cell membrane (Wachstein and Beier 1964, Kysle 1976). Moreover because inhibition of Na-K ATPase with cardiac glycosides greatly reduces both sodium reabsorption in the diluting segment (Martinez-Maldonado *et al.* 1970) and the metabolic rate of the outer medulla (Sejved *et al.* 1971, Lie *et al.* 1974). It is therefore possible that renal Na-K-ATPase can be stimulated by saline infusion.

Kilb (1971) proposed that sodium reabsorption in the diluting segment is raised during increased distal delivery of sodium because a rise in intracellular sodium concentration

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Renal Na K ATPase Activity during Saline Infusion in the Rabbit

By

OLE M. SEJERSTED

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Abstract

SEJERSTED, O. M. *Renal Na-K ATPase activity during saline infusion in the rabbit* Acta
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ing saline infusion, sodium reabsorption (R_{Na}) in the diluting segment (thick ascending limb of Henle) increased markedly. The mechanism for this higher pumping rate of outer medullary Na-K ATPase activity. Following left-sided nephrectomy, continuous infusion of hypertonic saline increased R_{Na} in the remaining, sole right kidney by 28 ± 14 ($p < 0.05$). Na-K ATPase activity in outer medulla increased by $(\Delta) 23 \pm 4$ above the left kidney ($p < 0.05$), whereas cortical activity was unchanged. The increase in Na-K ATPase activity was explored. The catalytic rate per enzyme did not differ in the two kidneys and equalled 5.340 min^{-1} . The increase was therefore due to higher tissue concentration of active enzymes. The response was fully developed during continuous infusion within 20 min. At equal magnitudes, further protein synthesis had been inhibited by cycloheximide ($\Delta = 23 \pm 7$) stimulated by unilateral nephrectomy 6 days earlier combined with saline infusion for 2 h ($\Delta = 34 \pm 10$). Thus, during hypertonic saline infusion, the increased R_{Na} in the outer medulla was partly accounted for the activation of latent Na-K ATPase. High delivery of sodium to the diluting segment for more than 2 h during hypertrophy caused no further activity change.

Key-words: Active sodium reabsorption, cycloheximide, enzyme activation, ion transport, kidney medulla, up of Henle, renal hypertrophy

Saline infusion increases both the sodium reabsorption in the thick ascending limb of Henle loop (diluting segment) (Rector *et al.* 1964, Schnemann 1968, Kunau *et al.* 1974) and the metabolic rate of the outer medulla (Kil *et al.* 1971). Several reports suggest that sodium reabsorption is carried out in this segment by Na-K-ATPase (Katz and Epstein 1967, Nectary and Nelson 1970), located at the peritubular cell membrane (Wachstein and Bean 1964, Kyte 1976). Moreover because inhibition of Na-K-ATPase with cardiac glycosides greatly reduces both sodium reabsorption in the diluting segment (Martinez Maldonado *et al.* 1970) and the metabolic rate of the outer medulla (Sejersted *et al.* 1971, Lio *et al.* 1974), it is therefore possible that renal Na-K-ATPase can be stimulated by saline infusion.

Kil (1971) proposed that sodium reabsorption in the diluting segment is raised during increased distal delivery of sodium because a rise in intracellular sodium concentration

would stimulate Na K ATPase. In addition to such direct substrate dependent stimulation the pumping rate could be increased due to higher catalytic rate for each enzyme (turnover number) even at constant sodium concentration or by a rise in the tissue enzyme concentration.

This study shows that in anesthetized rabbits the turnover number for renal Na-K-ATPase remained unchanged but that the concentration of active enzyme in the outer medulla increased by 23% during hypertonic saline infusion. This increment might be due either to synthesis of new enzyme protein or to recruitment of inactive enzyme. Experiments were therefore carried out to see whether the stimulation of Na-K-ATPase activity occurred rapidly to be accounted for by protein synthesis or whether the response was affected by inhibition of protein synthesis with cycloheximide.

It is well known that renal Na K ATPase activity increases after nephrectomy but this effect could be due to specific stimulation beyond the general stimulation of protein synthesis which can be attributed to hypertrophy (Katz and Epstein 1967). Hence, the effect of combination of unilateral nephrectomy and infusion of saline for 2 h 6 days later was compared to the increase in activity obtained after 20 min infusion.

The increment in Na K ATPase activity in outer medulla during hypertonic saline infusion probably does not fully account for the rise in sodium reabsorption in the distal segments but might be important in amplifying the effect of a rise in substrate concentration, i.e. the sodium concentration at the intracellular side of the peritubular membrane (Hall 1971).

Methods

Rabbits of both sexes weighing 1.5–3.2 kg were kept on a standard diet containing about 60 μ mol sodium and 450 μ mol potassium per gram. They were fasted overnight but had free access to water. Anesthesia was induced by sodium pentobarbital 30 mg/kg b.wt. (Nembutal), and maintained by additional doses of 5 mg/kg b.wt. administered through Venflon into an ear vein. Complete analgesia was obtained by subcutaneous injection of 70–80 mg lidocaine hydrochloride (Xylocain) before incisions were made. Free chest was exposed by tracheostomy and body temperature maintained with a heating lamp.

When clearance was determined, polyethylene catheters were inserted into a carotid artery and a jugular vein. The arterial catheter served for blood sampling and the pressure was recorded with a Statham pressure transducer (P23Gb) and a Sanborn amplifier and a recorder. All infusions were kept at body temperature. Both ureters were exposed through a midline abdominal incision and cannulated with soft polyurethane catheters. The incision was closed by towel clips.

Experimental procedure

The clearance of ^{51}Cr EDTA was determined as an estimate of glomerular filtration rate (GFR). ^{51}Cr EDTA was injected i.v. followed by a continuous infusion of 0.45% NaCl and 2.5% glucose at a rate of 22 μ l/min containing 30 μ Ci ^{51}Cr EDTA per ml, thus obtaining arterial concentrations of 3 000–6 000 cpm/ml. At least one hour elapsed before urine collection was started.

Acute experiments were performed in four groups of rabbits. In 6 rabbits which served as control group (Group I, Control), no clearance was determined. Through the abdominal incision the two kidneys were removed with a 20 min interval for enzyme analysis. In the second group (Group II, Acute Experiment, 7 rabbits), two control periods, with urine sampling for 30–60 min, elapsed before removal of the left kidney. Subsequently 13 ± 0.5 mmol/kg b.wt. sodium chloride in 1.45% solution (52 ml/kg b.wt.) was infused i.v. Half of the amount of saline was given in the course of 2–3 min, the rest at a rate of 3.5 ml/min. When three 1 min urine samples had been obtained, the right kidney was removed exactly 70 min after the left.

To test the effect of protein synthesis inhibition, the response to hypertonic saline was measured following

TABLE I. Effect of cycloheximide on the incorporation of ^3H -leucine into renal microsomes.

	Cortex dpm/mg prot.	Outer medulla dpm/mg prot.
Control	7980	6630
S.D.	± 440	± 440
Cycloheximide		
5 mg/kg body wt)	990	1960
S.D.	± 130	± 80
	0.05	<0.05

Animals had been unilaterally nephrectomized 4 days prior to the experiment. Following injection of cycloheximide or saline to the control group ^3H -leucine, 120 $\mu\text{Ci/kg}$ b.wt. was injected 1-3 h later the kidney was removed and analyzed. Mean values \pm standard error of the mean.

Cycloheximide 5 mg/kg b.wt. (Group III, Cycloheximide and Acute Expansion, 7 rabbits). The drug was injected 1-3 h following 2 control periods. Urine was collected for another 2 periods before the removal of the left kidney. Saline was infused as described above and right-sided nephrectomy performed after 20 min.

For control, the effect of cycloheximide on the rate of ^3H -leucine incorporation in renal microsomes tested in 10 separate rabbits (Group IV, Cycloheximide). Unilateral nephrectomy had been performed 7-10 days earlier to stimulate protein synthesis. The drug was injected 1-3 h in 5 rabbits and saline 1-3 h in others. Few were below 120 $\mu\text{Ci/kg}$ b.wt. ^3H -leucine was administered 1-3 h. The kidney was removed 3-5 min and radioactivity measured in the cortex and outer medulla. Microsomal tissue, prepared as described, was dissolved in 2 ml Soluene 350 (Packard) and added to 10 ml Dioxane (Packard) in counting vials. Incorporation was reduced by 85% in cortical tissue and 75% in outer medulla (Table I).

The effect of 6 days hypertrophy following left-sided nephrectomy combined with saline expansion for 4 h was tested in 5 rabbits (Group V, Nephrectomy and Prolonged Expansion). Following the hypertrophy period, 45 mmol sodium chloride as administered 8-9 mmol as 1.45 solution (32-36 ml) in the course of 1-2 h, the rat in continuous sodium infusion, ^{51}Cr EDTA clearance was measured only in the right kidney before and during the infusion. Enzyme activities were compared in the left and right kidneys.

The ^3H activity was measured in an LKB-Wallac 81.30 liquid scintillation spectrometer and ^{51}Cr EDTA activity in a Beckman LS 5000TD counter. Sodium and potassium were measured by flame photometer using lithium as the internal standard (Instrumentation Laboratories 343).

Tissue preparation and enzyme analysis

Following removal, the kidneys were quickly compressed and thereby drained for blood and urine before immersion in ice-cold buffer containing 30 mmol/l histidine and 230 mmol/l sucrose at pH of 7.2 at 20°C (histidine-sucrose buffer). During the following procedures the tissue was kept at 0°C.

Tissue from cortex and outer medulla was homogenized separately in histidine-sucrose buffer (10 ml) in Potter-Elvehjem Teflon/glass homogenizer. Crude homogenate was obtained by ultrafiltration of 2 ml of the suspension in small glass tube with wall/pore clearing of 0.11-0.15 μm . The microsomal fraction was prepared as described by Jørgensen and Skov (1971). Following centrifugation (50 000 g, 30 min) the pellet was resuspended in buffer homogenized, and centrifuged again. The supernatant was then spun for 1.5 $\times 10^6$ g, 30 min, the pellet suspended in buffer and regarded as the microsomal fraction. These enzyme preparations could be stored frozen for weeks without loss of Na-K ATPase activity. However, during preparation of microsomes, more than 90% of the tissue protein was discarded and of the total enzyme activity in the crude homogenate, variable fraction of about 45% remained. As consequence, there is poor correlation between activities measured in crude homogenate and microsomes (Fig 1). In this study enzyme activity was therefore measured as rate of ATP hydrolysis per mg protein in the crude homogenate.

Enzyme activity was measured simultaneously in the two kidneys from the same rabbit by the method described by Jørgensen and Skov (1971). The enzyme was preincubated in 25 $\mu\text{mol/l}$ EDTA, 1.7 $\mu\text{mol/l}$ Na-docosate (NaDOC) (mmol/l), pH 7.0 for 30 min at 20°C. This concentration of NaDOC produced the highest enzyme activity. Of the crude homogenate, 0.5-1.0 mg protein was preincubated in volume of

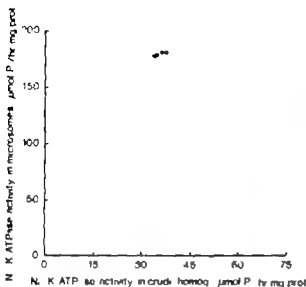


Fig. 1 Comparison of Na-K-ATPase activity in crude homogenate and microsomes from outer medulla in 33 tubes. The correlation is significant ($t=3.0$, $0.005 < p < 0.01$) but only 11% of variation in microsomal activity is explained by the variation in homogenate activity.

1 ml and similarly 10–40 μ g protein from the microsomes in a volume of 0.1 ml. 25 μ l of the reaction was transferred to 1 ml of the incubation medium containing 30 histidine, 3 Tris-ATP, 2MgCl₂ (W), 20 KCl, 1 phosphoenolpyruvate (PEP) (mmol/l) and 30 μ g pyruvate kinase (PK). The reaction is stopped after 10 min at 37°C with 0.1 ml 30% trichloroacetic acid (TCA). Measurements were taken in duplicate and Na-K-ATPase activity calculated on the basis of the difference in ATP hydrolysis rate in the absence and presence of ouabain, 1 mmol/l. Data for Mg-ATPase are not included because this enzymatic activity is unstable on preparation and storage (unpublished observation).

Inorganic phosphate was determined according to Fiske and Subbarow (1925) using AMIDOL (B. Chemicals Ltd.) as a reducing agent. Protein was determined in the enzyme preparations with the Folin reagent (Merck) and modified Lowry *et al.* (1951). A standard curve was obtained by serial dilution of enzyme preparation where the protein content had been measured by the biuret method. Bovine serum albumin (Sigma Chemical Company) dissolved in histidine-mucosa buffer was used as standard. Photometric readings were carried out in Beckman DU spectrophotometer.

The ionic concentrations in the incubation medium were chosen to give maximal enzyme activity (Fig. 2). Tris-ATP was prepared from the disodium salt of "Sigma grade" ATP (Sigma Chemical Co.).

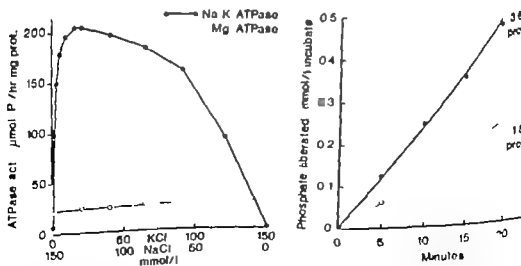
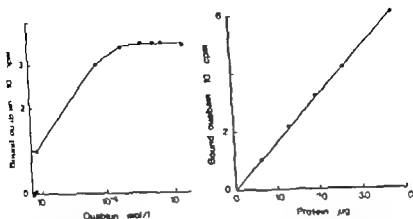


Fig. 2 Incubation characteristics of outer medullary microsomes. Left: Effect of potassium concentration. Right: Reaction rate is linear and independent of the protein concentration.



1. Binding of 3H -ouabain to microsome Na-K-ATPase from outer medulla. Left: Effect of varying the ouabain concentration. Right: Standard curve with ouabain concentration $4 \times 10^{-6} \text{ mol/l}$.

in ion exchange columns (Amberlite Rumn IR 120, Standard grade, BDH Chemicals Ltd). Less than satisfactory effect of this preparation could be observed in contrast to the great inhibition reported for the disodium salt (Charney *et al.* 1975). Apparent K_m for Tris ATP was found to be less than 0.5 μM .

Turnover rate was constant even at concentrations of split phosphatase reaching 20% of the original ATP maximum (Fig. 2) both as kept constant by the PEP/CK system.

Assay of ouabain binding

Binding of ouabain to microsome from outer medulla was used as an estimate of ouabain concentration. According to Jørgensen (1974), the number of ouabain binding sites equals the number of ATP binding sites and therefore assumed binding of one mole ouabain to one mole enzyme. Enzyme activity per mass binding site is then an estimate of the turnover number.

The saturation technique was preferred because much more tissue was required to obtain acceptable Michaelis plots, more than could be obtained from the entire outer medulla of one rabbit kidney.

Following preincubation as described, 100 μl (30–150 μg protein) are transferred to tubes containing 10^{-4} 3H -ouabain, 30 Tris-HCl, 3 Tris-ATP, 5 MgCl_2 , 120 NaCl (mmol/l) in final volumes of 1 ml at pH 7.4 at 25°C , and incubated for 2 h at 37°C . Unspecific binding was measured following incubation with only 3H -ouabain and Tris-HCl. The incubate was then filtered by means of Millipore filters (GKWP 300), washed with 15 ml ice-cold Tris-HCl 30 mmol/l, dried at 80°C , and counted in vials containing 10 lubeater with 5 g PPO and 0.05 g POPOP per litre.

Filters with pore size 0.45 μm were found to retain all measurable Na-K-ATPase activity. Binding of ouabain was maximal and stable after 60 min incubation. Binding was dependent on the MgCl_2 concentration, but maximal at concentrations above 3 mmol/l. As evident from Fig. 3, maximal binding was obtained at ouabain concentrations above $2.0 \times 10^{-6} \text{ mmol/l}$ and $4 \times 10^{-6} \text{ mmol/l}$ as chosen as the standard concentrations. The linearity of the standard curve (Fig. 3) indicates that this concentration was adequate for maximal binding at the range of protein indicated. The concentration is at least 100 times higher than the maximal concentration of ouabain binding sites. Consequently less than 1% of the ouabain was bound to enzyme equilibrium kinetics. This can be calculated that even with dissociation constant of about 10^{-8} mol/l , which is much higher than reported in other studies (Jørgensen 1974), more than 99% of the ouabain binding sites would be occupied.

3H -ouabain (Amersham) was purified by extraction with microsomes from dog renal outer medulla (about 80% of the activity was extractable) and distilled with unlabelled ouabain (Merck) to give 30% cpm nmol (about 0.34 Ci/nmol) when counted bound to enzyme on the filters. With this counting system quenching was constant.

Crude homogenates could not be used for binding measurements because the filters got jammed. At chemical incubation conditions, the turnover number of the enzyme population is assumed homogeneous and the microsome Na-K-ATPase therefore representative.

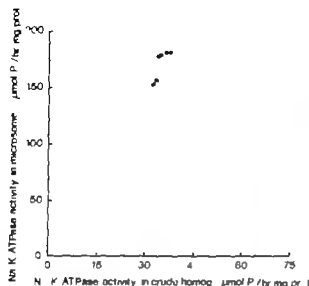


Fig. 1 Comparison of Na-K-ATPase activity in crude homogenate and microsomes from outer medulla is 30 later. The correlation is significant ($r=0.8$, $0.005 < p < 0.01$) but only 15% of the variation in microsomal activity is explained by the variation in homogenate activity.

1 ml and similarly 10–40 μ g protein from the microsomes in a volume of 0.1 ml. 25 μ l of the preparation was transferred to 1 ml of the incubation medium containing 30 histidine, 3 Tris-ATP, 2MgCl₂, 130 mM KCl, 1 phosphoenolpyruvate (PEP) (mmol/l) and 30 μ g pyruvate kinase (PK). The reaction is stopped after 10 min at 37°C with 0.1 ml 50% trichloroacetic acid (TCA). Measurements were taken in duplicate and N-K ATPase activity calculated on the basis of the difference in ATP hydrolysis rate in the absence and presence of ouabain, 1 mmol/l. Data for Mg-ATPase are not included because this enzymatic assay is unstable on preparation and storage (unpublished observation).

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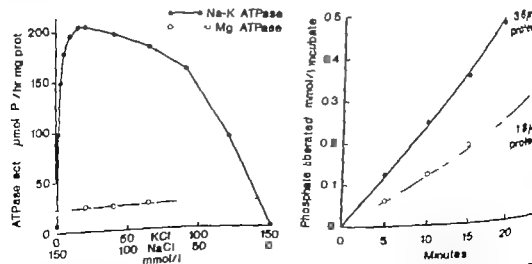


Fig. 2. Incubation characteristics of outer medullary microsomes. Left: Effect of alterations in sodium and potassium concentration. Right: Reaction rate is linear and independent of the protein concentration.

TABLE III. Effect of 10% saline infusion on blood pressure, glomerular filtration rate, sodium excretion and reabsorption, and potassium excretion in Groups II, III and V

	BP mmHg	GFR ml/min		P_{Na} mmol/l	$U_{Na}V$ mmol/min		Na reab mmol/min		P_K mmol/l	U_KV mmol/min	
		left kidney	right kidney		left kidney	right kidney	left kidney	right kidney		left kidney	right kidney
Group II											
control	76	2.9	3.1	142	1.9	1.8	410	438	2.2	1.9	2.0
	± 4	± 0.5	± 0.6	± 2	± 0.6	± 0.7	± 70	± 80	± 0.1	± 0.3	± 0.3
2h Expansion	84		8.8	154		453		562	1.8		8.1
	± 6		± 0.6	± 2		± 73		± 53	± 0.1		± 1.4
Group III											
control	79	4.0	3.8	144	4.0	3.8	372	342	2.3	2.7	2.6
	± 6	± 0.5	± 0.5	± 1	± 1.6	± 1.6	± 77	± 77	± 0.2	± 0.3	± 0.4
2h cycloheximide	80	4.8	4.3	143	3.4	3.0	687	622	2.6	2.7	2.3
	± 7	± 1.0	± 0.7	± 2	± 1.4	± 1.1	± 136	± 105	± 0.3	± 0.5	± 0.4
2h cycloheximide Acute Expansion	91		7.2	158		495		639	2.1		9.4
	± 7		± 0.8	± 2		± 62		± 81	± 0.2		± 1.6
Group V											
4h nephrectomy	76		4.7	146		3.0		602	2.9		4.0
	± 8		± 1.2	± 2		± 0.7		± 167	± 0.2		± 0.6
4h nephrectomy Prolonged Expansion	96		7.5	158		228		925	2.6		7.8
	± 7		± 0.8	± 2		± 0.2		± 123	± 0.2		± 0.3

BP blood pressure GFR glomerular filtration rate $U_{Na}V$ sodium excretion U_KV potassium excretion, P_{Na} plasma sodium concentration P_K plasma potassium concentration. Mean values \pm standard error of the mean.

The response of outer medullary Na-K-ATPase activity to hypertonic saline infusion was unaffected after inhibition of the renal protein synthesis (cycloheximide, 5 mg/kg b.wt., Table II, Group III) with $23 \pm 7\%$ increase in enzyme activity. In this group, GFR was raised by 11% and fractional sodium reabsorption lowered to $56 \pm 3\%$. Sodium reabsorption was not significantly changed, but the natriuretic response was similar in the group with intact protein synthesis (Table III).

Saline infusion might possibly change the sensitivity of renal tissue to NaDOC. In Fig. 4 it is shown that the enzyme activity in homogenate from outer medulla was $30 \pm 3\%$ (SD) higher in the left kidney at all concentrations of NaDOC.

Effect of nephrectomy and prolonged saline expansion (Group V)

During a postnephrectomy period of 6 days, the dry weight of the left kidney increased 17.4% above the right. Combined with hypertonic saline infusion for 2 h the hypertrophy raised outer medullary Na-K-ATPase activity by $34 \pm 10\%$, not significantly more than during acute saline expansion (Table II). Cortical activity remained unchanged.

TABLE II Effect of acute and prolonged saline expansion on Na K ATPase activity in the cortex and outer medulla of rabbit kidneys.

	Cortex			p	Outer medulla		
	Na K ATPase act., $\mu\text{mol P}_i/\text{hr mg prot.}$		Ratio right/ left		Na K ATPase act., $\mu\text{mol P}_i/\text{hr mg prot.}$		Ratio: right/ left
	left kidney	right kidney			left kidney	right kidney	
Group I Control (n = 6) p	23.3 ± 1.5 -0.05	23.6 ± 1.6	1.06 ± 0.03		32.3 ± 1.3 NS	32.9 ± 3.3	1.03 ± 0.10
Group II Acute Expansion (n = 7) p	25.2 ± 2.1 -0.05	27.3 ± 2.0	1.08 ± 0.04	NS	27.3 ± 2.6 <0.05	33.6 ± 2.8	1.23 ± 0.04
Group III Cycloheximide and Acute Expansion (n = 7) p	27.8 ± 1.4 NS	29.3 ± 1.3	1.05 ± 0.06	NS	29.9 ± 1.7 <0.05	36.7 ± 1.5	1.23 ± 0.07
Group V Nephrectomy and Prolonged Expansion (n = 5) p	27.9 ± 2.4 NS	31.7 ± 1.5	1.14 ± 0.09	NS	45.6 ± 3.4 -0.05	61.2 ± 4.9	1.34 ± 0.10

Left kidney: control; right kidney: experimental. Mean values \pm standard error of the mean. p: Wilcoxon signed rank test for paired comparisons. p: p-values for comparison with the ratios of the control (Group I) with Wilcoxon signed rank test.

Statistics

All values are given as means \pm standard errors of the means (S.E.), when not otherwise stated ratios were calculated according to Cameron (1960).

Wilcoxon signed rank test for paired comparisons was used to evaluate differences between left kidneys, whereas groups were compared with the ordinary Wilcoxon signed rank test.

Regression was calculated by the method of least squares and probability of correlation by χ^2 tested by t statistics (Hodges and Lehmann 1970).

Results

Effect of 1.5% infusion of 1.45% saline for 20 min

During control conditions, Na K ATPase activity for the whole kidney averaged $\mu\text{mol P}_i/\text{g min}$ and the sodium reabsorption, $53 \pm 8 \mu\text{mol/g min}$ (wet kidney). Following hypertonic saline infusion, Na K ATPase activity was not significantly in cortex but was higher by $23 \pm 4\%$ in outer medulla (Table II group II). The hypertonic saline infusion raised plasma sodium concentration by 12 mmol/l, doubled GFR, and the fractional sodium reabsorption increased by $28 \pm 14\%$ ($p < 0.05$) (Table III). Fractional reabsorption of sodium fell to $57 \pm 5\%$, whereas the fractional excretion of potassium increased from $30 \pm 3\%$ to $71 \pm 11\%$ despite a fall in plasma potassium concentration (Table III).

TABLE III. Effect of 1.45% saline infusion iv on blood pressure, glomerular filtration rate, sodium excretion and reabsorption, and potassium excretion in Groups II, III and V

	BP mmHg	GFR ml/min		P_{Na} mmol/l	$U_{Na} V$ mmol/min		Na reab mmol/min		P_K mmol/l	$U_K V$ mmol/min	
		left kidney	right kidney		left kidney	right kidney	left kidney	right kidney		left kidney	right kidney
Group II											
intact	76 ±4	88 ±0.5	3.1 ±0.6	142 ±2	19 ±0.6	18 ±0.7	410 ±70	430 ±80	2.2 ±0.1	1.9 ±0.3	2.0 ±0.3
adrenalectomy	84 ±6		6.6 ±0.6	154 ±2		453 ±73		562 ±53	1.8 ±0.1		8.1 ±1.4
Group III											
intact	79 ±6	88 ±0.5	3.8 ±0.5	144 ±1	4.0 ±1.6	3.8 ±1.6	572 ±77	542 ±77	2.3 ±0.2	2.7 ±0.3	2.6 ±0.4
adrenalectomy	80 ±7	4.8 ±1.0	4.3 ±0.7	143 ±2	3.4 ±1.4	3.0 ±1.1	687 ±138	622 ±105	2.6 ±0.3	2.7 ±0.3	2.5 ±0.4
adrenalectomy acute expansion	91 ±7		7.2 ±0.8	158 ±2		495 ±6		639 ±82	2.1 ±0.2		9.4 ±1.6
Group V											
adrenalectomy	76 ±8		4.7 ±1.2	146 ±2		3.0 ±0.7		682 ±167	2.9 ±0.3		4.0 ±0.6
adrenalectomy Prolonged Expansion	98 ±7		7.5 ±0.8	158 ±2		228 ±62		925 ±123	2.6 ±0.1		7.8 ±0.3

BP = blood pressure, GFR = glomerular filtration rate, $U_{Na} V$ = sodium excretion, $U_K V$ = potassium excretion, P_{Na} = plasma sodium concentration, P_K = plasma potassium concentration. Mean values \pm standard error of the mean.

The response of outer medullary Na-K-ATPase activity to hypertonic saline infusion was unaffected after inhibition of the renal protein synthesis (cycloheximide, 5 mg/kg b.wt., Table II, Group III) with $23 \pm 7\%$ increase in enzyme activity. In this group, GFR was raised by 67% and fractional sodium reabsorption lowered to $56 \pm 3\%$. Sodium reabsorption was not significantly changed, but the natriuretic response was similar in the group with intact protein synthesis (Table III).

Saline infusion might possibly change the sensitivity of renal tissue to NaDOC. In Fig. 4 it is shown that the enzyme activity in homogenate from outer medulla was $30 \pm 3\%$ (SD) higher in the left kidney at all concentrations of NaDOC.

Effect of nephrectomy and prolonged saline expansion (Group V)

During a postnephrectomy period of 6 days, the dry weight of the left kidney increased 17.4% above the right. Combined with hypertonic saline infusion for 2 h the hypertrophy raised outer medullary Na-K-ATPase activity by $34 \pm 10\%$, not significantly more than during acute saline expansion (Table II). Cortical activity remained unchanged.

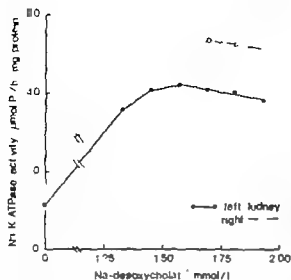


Fig. 4. Comparison of the effect of protein with Na-desoxycholate on outer medullary homogenate from the left (control) and the (hypertonic saline infusion for 20 min) kidney in one rabbit.

In the hypertrophied kidney sodium reabsorption was 37% higher ($p < 0.05$) than in control conditions in the group acutely expanded (Table II). Prolonged saline infusion raised sodium reabsorption further by $36 \pm 18\%$ (Table III) parallel to $58 \pm 30\%$ increase in GFR.

Binding of ouabain to microsomal Na-K ATPase from outer medulla

The average turnover number *i.e.* maximal catalytic rate per active site, for all animals ouabain binding was measured ($n = 21$) equalled 5250 ± 340 min⁻¹ in the left kidney, 5430 ± 330 min⁻¹ in the right experimental kidney and there was no difference between two kidneys in any experimental group (Fig. 5). Thus the increase in activity during infusion is caused by a higher concentration of enzyme.

From Table IV it is evident that the binding of ouabain to microsomal protein averaged 0.35 ± 0.01 nmol/mg protein ($n = 42$). Binding of ouabain was higher in the right than

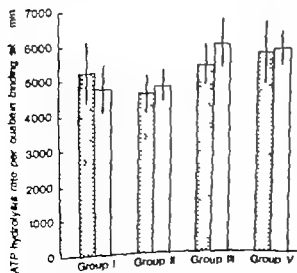


Fig. 5. Estimates of the maximal catalytic rate for the Na-K ATPase from the (dotted columns: control) and right (solid columns: experimental) kidneys in the experimental groups. Group I: In this group the left kidney was removed 2 weeks before the right. Group II: Following bilateral nephrectomy hypertonic saline was infused for 20 min before the right kidney was also removed. Group III: As group II except that cycloheximide 5 mg/kg was infused prior to removal of the kidney. Group V: Six days followed by hypertonic saline infusion for 2 h elapsed before the two nephrectomies.

TABLE IV Binding of ouabain to microsomes from rabbit renal outer medulla.

	Group I Control	Group II Acute expansion	Group III Cyclohex. + acute expansion	Group V Nephrect. + prolonged expansion
kidney (control)	0.37 ± 0.04	0.33 ± 0.03	0.32 ± 0.02	0.37 ± 0.02
pk kidney (experimental)	0.35 ± 0.03	0.37 ± 0.03	0.34 ± 0.02	0.45 ± 0.02
	NS	0.05	NS	
	(n = 5)	(n = 6)	(n = 7)	(n = 3)

pmol ouabain/mg protein. Mean values \pm standard error of the mean.

in the left kidney only in the acutely infused group (Group II), but the microsomal Na-K ATPase activity was increased in parallel leaving the turnover number unchanged. Jørgensen (1974) reports a higher turnover number probably due to the higher purity of preparation and different method of activation.

Discussion

The combined effects of extracellular volume expansion and a rise in plasma sodium concentration increase the Na-K-ATPase activity of outer medulla by $23 \pm 4\%$, whereas cortical activity is unchanged. Katz and Genant (1971) showed originally that the activity of rat kidney enzyme increased by 12.5% in response to i.v. isotonic saline infusion for 3 h, but in a later report, ascribed this to an artifact because the results were divergent when analysing the microsomal fraction (Katz and Lindheimer 1975). This study demonstrates that the functional significance of Na-K-ATPase activity is best expressed in relation to total kidney protein or mass, whereas the high specific activity obtained in the microsomal fraction only represents a variable fraction of total renal enzyme activity resulting in a poor correlation between activities in the crude homogenate and the microsomal fraction. Furthermore, changes in tissue enzyme concentration can not be detected if a highly purified preparation is analysed even if all the activity is recovered. The present experimental design allows paired comparison, and the extreme hypertonic extracellular expansion ensures a great increase in sodium reabsorption in the outer medulla (Kilb *et al.* 1971).

The enzymatic response to saline infusion developed within 20 min. Maximal catalytic rate per active site was not increased, as judged from the ratio of enzyme activity to bound ouabain in the microsomal fraction. Consequently the tissue concentration of enzyme was raised by either activation of latent enzymes or by rapid synthesis of new protein. The latter possibility is ruled out as the enzymatic response to saline infusion was unaffected following inhibition of protein synthesis at the ribosomal translation level with an effective dose of cycloheximide. The rapid adaptation of outer medullary Na-K-ATPase is thus due to an increase in the number of pumping sites by recruitment of inactive enzymes.

The mechanism of acute Na-K-ATPase activation might involve activation through protein kinase, association of the subunits, or some unspecific change of membrane configuration. However if the effect was denaturation of intact enzymes, inaccessible to

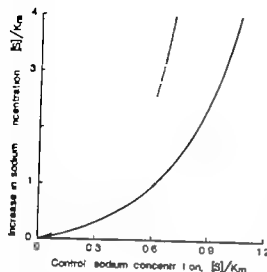


Fig. 6. Based on Michaelis-Menten kinetics it has been calculated what the rise in intracellular sodium concentration $\Delta S/K_m$ would have to be to obtain a doubling of the Na-K ATPase pumping rate at different concentrations S/K_m . The broken line is calculated assuming unchanged V_{max} , whereas the unbroken line takes into account a concomitant 23% increase of V_{max} .

substrate, NaDOC treatment would be expected to abolish the difference between control and expanded kidneys. During homogenization, the plasma membrane forms vesicles which may be opened by NaDOC treatment, thereby increasing the *in vitro* enzyme activity. No similar protective mechanism of the Na-K ATPase exists *in vivo* as the difference in enzyme activity between the two kidneys was independent of the NaDOC effect.

It is not possible to quantify the change in sodium reabsorption in the diluting segment from the present data. Findings from micropuncture studies on rats (Landwehr *et al* 1967, Kunau *et al* 1974) support measurements in dogs of both free water clearance (Rector *et al* 1964) and renal metabolic rates (Kill *et al* 1971) showing that sodium reabsorption in outer medulla may be raised more than in other parts of the kidney during saline infusion. Katz and Lindheimer (1975) argue that an estimated increase of 100–200% in sodium reabsorption in the outer medulla cannot be accounted for by the much smaller increase in Na-K-ATPase activity. However, as discussed in the following, an activation of latent enzymes amounting to 23% in combination with an elevation of the intracellular sodium concentration can cause a great increase in the cellular pumping rate of sodium.

As proposed by Kill (1971), the luminal sodium concentration in outer medulla will be elevated during increased delivery of sodium to the diluting segment. Thus, intracellular sodium concentration will also be increased stimulating the Na-K ATPase in the peritubular membrane. A new steady state is reached where net flux of sodium through the cell is elevated and intracellular sodium concentration maintained at a higher level.

The *in vitro* activation of Na-K ATPase by sodium does not follow Michaelis-Menten kinetics because of allosteric interaction (Lindenmayer *et al* 1974), but the deviation is slight and the response to sodium may be described by an apparent K_m and V_{max} (Katz and Lindheimer 1975). If saline expansion did not change the number of pumping sites (V_{max}), intracellular sodium concentration should be well below K_m beforehand to obtain a doubling of the pumping rate. Even so, at a starting concentration of $0.61 K_m$, the intracellular sodium concentration would have to rise by $2.5 K_m$ (Fig. 6, circle on broken line). However, taking into account the 23% increase in enzyme activity (23% increase in V_{max}) it is necessary to increase the intracellular concentration of sodium only $1 K_m$ (Fig. 6).

de on unbroken line) to obtain the same doubling of the pumping rate. Hence, a small rise in the number of pumping sites amplifies the response to a rise in intracellular ion concentration. An implicit condition is that the enzymatic response to variations in ion concentration is identical before and after saline infusion, which has been demonstrated by others (Katz and Lindheimer 1975).

A consequence of this hypothesis is that effective intracellular sodium concentration must be higher than K_m during hydropenic conditions if a doubling of the sodium reabsorption is to occur in response to increased delivery of sodium to the distal nephron. As the action of Na-K-ATPase to sodium is influenced by other cations such as Mg^{2+} , Ca^{2+} and K^{+} (Skoog 1965, Lindemeyer *et al.* 1974), no data are available on the *in vivo* K_m . Intracellular concentrations of sodium and potassium could vary inversely affecting enzyme activity as shown in the right part of the curve in Fig. 2. However reliable estimates of intracellular ion concentrations in the renal outer medulla have not been made. As can be deduced from Fig. 6, the response to an increment in intracellular sodium concentration is greater the lower the starting concentration.

The total catalytic capacity of the whole kidney for ATP hydrolysis by Na-K-ATPase is of the same order of magnitude as the sodium reabsorption during control conditions, assuming that 2-3 sodium ions are transported per ATP hydrolyzed (Sejersted *et al.* 1976), a transport rate is 30-50% of maximum providing renal Na-K-ATPase takes care of all sodium reabsorption. This does not hold true, especially in the proximal tubules where the bulk of reabsorbed sodium could pass through paracellular pathways (Mathiesen *et al.* 1976). In the distal segment, however where the Na-K-ATPase activity is highest (Schmidt and Dubach 1969), less than half of the filtered sodium load is reabsorbed and the enzymes therefore, must be working at less than 30% of their maximal catalytic rate.

The maximal enzymatic response to an increase in filtered load of sodium is $34 \pm 10\%$ higher specific activity of the outer medullary Na-K-ATPase following nephrectomy in combination with prolonged saline infusion. This is not significantly more than obtained by 30 min hypertonic saline infusion. Katz and Epstein (1967) reported a 25% increase in light microscopic Na-K-ATPase from whole rat kidneys 7 days postnephrectomy. The present results show that this increase is confined to the outer medulla and that saline infusion, on top of the response to nephrectomy produces, if anything, only a very small further activation of latent enzymes. Thus, following unilateral nephrectomy the activation of renal Na-K-ATPase might not be due to the hypertrophy *per se* but might involve an activation of latent enzymes similar to the response obtained by acute increase in delivery of sodium to the distal segment.

Because dry renal weight increased by $17 \pm 4\%$ following nephrectomy and the amount of renal protein even more, as evidenced by higher protein concentration (Malt 1969), how the Na-K-ATPase activity is expressed is of the utmost importance. Following nephrectomy and saline infusion, the total number of renal Na-K-ATPase molecules is thus increased much more than 50%. Schmidt and Dubach (1974) observed very rapid elevation of cortical distal tubular Na-K-ATPase activity in relation to tubular dry weight which, compared to the unaltered cortical activity found in the present study must at least partly be ascribed to the smaller increment in tissue weight compared to the amount of total tissue protein.

Many factors, including dietary potassium or protein intake (Silva *et al* 1973, Field and Hayslett 1975, Katz and Epstein 1967), or administration of glucocorticoids (Ma *et al* 1968, Katz and Epstein 1967) seem to be important determinants for the regulation of renal Na K ATPase activity. So far the only argument against a relation between sodium reabsorption in the diluting segment and Na K ATPase is that activity remained unchanged when GFR was acutely or chronically reduced by clamping of the renal artery (Nechay and Nelson 1970, Fisher *et al* 1975). In this situation, the renal sodium reabsorption could be due to lower intracellular sodium concentration.

At least two mechanisms may account for acute alterations in sodium reabsorption in the diluting segment. Firstly alterations in intracellular sodium concentration may directly affect the catalytic rate of renal Na K ATPase. Secondly latent enzyme is activated by saline infusion thereby increasing the concentration of active Na-K ATPase in the medulla.

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Effect of Sodium Pentobarbital on the Apparent Turnover of Acetylcholine in Different Brain Regions

By

AGNETA NORDBERG and ANDERS SUNDWALL

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Abstract

NORDBERG A. and A. SUNDWALL. *Effect of sodium pentobarbital on the apparent turnover of acetylcholine in different brain regions* Acta physiol. scand. 1977 99 336-344

The turnover rate of acetylcholine (ACh) was measured in six different brain regions in the mouse by pulse injection of radioactive choline (Ch) and killing of the animals by microwave irradiation of it (0.25 s, 5 kW). The time course of the change in $^3\text{H-ACh}/^3\text{H-Ch}$ ratio was linear 0-60 s in all brain after administration of $^3\text{H-Ch}$. Plots of the specific radioactivities (SA) of ACh and Ch versus time (precursor-product relationship) in all brain regions except the cerebellum both in control and pentobarbital anesthetized animals. The turnover was highest in the striatum (55 nmol $\text{g}^{-1} \text{min}^{-1}$) in the cortex and hippocampus this value was approximately half (27 and 21 nmol $\text{g}^{-1} \text{min}^{-1}$) in midbrain and medulla oblongata the turnover rates were only 11 and 10 nmol $\text{g}^{-1} \text{min}^{-1}$. Sodium barbital anesthesia reduced specifically the turnover in the cortex and hippocampus to about 60 per cent.

Key words: Turnover acetylcholine, choline, brain regions, sodium pentobarbital, microwave irradiation.

Barbiturate anesthesia increases the steady state level of endogenous acetylcholine (Tobias *et al.* 1946, Richter and Crossland 1949) and decreases its turnover in the brain (Schubert *et al.* 1969). Studies of the effect of sodium pentobarbital on the state level of ACh in different brain regions have shown that barbiturates markedly increase the ACh level in the hippocampus and cerebral cortex, slightly in the midbrain but practically no change in the cerebellum, medulla oblongata and striatum (Nordberg and Sundwall 1973-1975). The biosynthesis of radioactive ACh is also most markedly increased in the hippocampus and cortex. The turnover could not be estimated, however, on a post mortem increase in endogenous choline (Ch).

The present paper describes experiments in which mice were killed by microwave irradiation, a technique which produces rapid enzyme inactivation and at the same time allows a rapid dissection of the brain (Stravinsky *et al.* 1970-1974, Nordberg and Sundwall 1975). Both endogenous Ch and ACh as well as the rate of biosynthesis of radioactive ACh

measured in different brain regions. The results show that the ACh turnover differs markedly between different brain regions and is specifically decreased in the cerebral cortex and hippocampus following treatment with sodium pentobarbital.

Materials and Methods

Male mice (NMRI strain) weighing 20-24 g. are supplied with food and water *ad libitum* and kept under short diurnal lighting (artificial) for at least 6 days. The light is switched on at 6 a.m. and switched off at 6 p.m.

The animals are always killed between 10 a.m. and 11.30 a.m. Sodium pentobarbital (60 mg/kg) is saline as injected intraperitoneally (i.p.) and 15 min later an i.p. injection of 15 μ mol of 3 H-Me-Ch (16.5 Ci/mmol) dissolved in 100 μ l of saline is given to 2-3 into tail.

The rectal temperature is kept at 38°C by warming the animals with an infrared lamp. At different periods after the injection of 3 H-Me-Ch (0.5, 1.5 or 10 min) the animals are killed by microwave irradiation of the head for 0.25 (5 kW 2.450 MHz, Metabostat, Gerling Moore) (Nordberg and Sundvall 1976). Animals injected with saline instead of sodium pentobarbital served as controls.

The brains were rapidly removed, placed on an ice-cold glass plate and dissected into six discrete regions: cerebellum, medulla oblongata + pons, midbrain (including hypothalamus), striatum, hippocampus and cortex essentially according to the method described by Glowinski and Iversen (1966). The brain tissue was weighed, homogenized and extracted with 7 ice-cold trichloroacetic acid (TCA). The homogenates were stored in cold room (-5°C) for 30 min and centrifuged at 4 000 rpm for 10 min (for cortex 6 000 rpm, 15 min). The pellets were resuspended with TCA and centrifuged. To remove excess TCA the combined supernatants were extracted with portions of ether until pH of 4.0 was reached. The extracts are freeze-dried and dissolved in 100 μ l glass-distilled ether. A sample (20 μ l) of the extract is used for determination of the total radioactivity (3 H-tar) and the same volume was submitted to high voltage electrophoresis.

Separation of the principal metabolites: H-phosphorylcholine (PbCh), 3 H-ACh and 3 H-Ch. The buffer solution contained pyridine, acetic acid, acetic anhydride and water (8:8:30:154 v/v). After scanning the paper with a Packard Radiochromatograph the radioactivity is measured by liquid scintillation. 10 μ l of the extract is used for determination of endogenous ACh using the dorsal muscle of the leech suspended in a beaker (Nordberg and Sundvall 1976).

Endogenous Ch was extracted to separate homogenates. The brain regions were homogenized and extracted with 1 M formic acid and acetone (15:85 v/v), kept in cold room for 30 min and centrifuged at 300 rpm for 15 min. The supernatants were extracted 3 times with heptane and chloroform (80:20 v/v). The organic and lipid phases are carefully separated. Ch as separated by low voltage electrophoresis, with 20 formic acid, glacial acetic acid and water (25:11:900 v/v), eluted from the paper strips and labelled with 3 H-acetyl-CoA and choline acetyltransferase according to modification (Nordberg and Sundvall 1976) of the method of Sjöström *et al.* (1973).

Results

1. Effect of sodium pentobarbital on endogenous ACh and Ch in discrete brain regions

As seen in Table I that sodium pentobarbital (60 mg/kg, i.p.) increased the endogenous steady state level of ACh by about 90% in the hippocampus, 70% in the cortex and about 30% in the midbrain. No effect was noted in the striatum, medulla oblongata or cerebellum. In contrast, endogenous Ch was fairly evenly distributed and no significant effect was observed with the exception of an increase in the cerebellum ($p < 0.05$).

2. Effect on biosynthesis of H-Ch in discrete brain regions

The results are summarized in Fig. 1-4. As seen in Fig. 1 sodium pentobarbital decreased ($p < 0.001$) the initial rate of biosynthesis of 3 H ACh in the hippocampus (by about 75%), cortex (about 65%) and midbrain (about 40%).

TABLE 1 Effect of sodium pentobarbital (60 mg/kg i.p.) on the steady state levels of endogenous choline and acetylcholine (nmol g⁻¹) in discrete mouse brain regions. The animals were killed by microwave irradiation of the head (0.25 s, 5 kW) 16 min after administration of sodium pentobarbital

Brain region	Choline		Acetylcholine	
	Control	Sodium pentobarbital	Control	Sodium pentobarbital
Cerebellum	32.8 ± 4.63 (10)	47.3 ± 3.49 (8)	5.2 ± 1.05 (4)	5.7 ± 0.73 (4)
Medulla oblongata	43.8 ± 4.30 (8)	37.2 ± 4.89 (4)	29.1 ± 1.10 (4)	32.5 ± 3.10
Midbrain	33.8 ± 3.56 (9)	44.7 ± 7.80 (7)	29.0 ± 1.80 (6)	35.5 ± 1.77 (4)
Striatum	38.1 ± 3.11 (10)	45.9 ± 6.30 (9)	76.8 ± 1.78 (5)	81.3 ± 12.10
Hippocampus	48.4 ± 3.11 (8)	32.2 ± 3.69 (8)	21.2 ± 1.32 (6)	39.8 ± 8.23 (4)
Cortex	33.8 ± 3.34 (11)	39.0 ± 2.26 (9)	24.8 ± 1.44 (6)	41.1 ± 1.27 (4)

Mean value (M) ± S.E. (n) = number of expts. $p < 0.001$ $p < 0.05$.

In the cerebellum, medulla oblongata and striatum. The H-Ch concentration was 25% higher in the cortex and hippocampus than in the controls (Fig. 2).

The rate of H PhCh biosynthesis was increased at 1 min in the cortex and hippocampus (Fig. 3).

The time course of the change in the ³H ACh/³H-Ch ratio was linear at 0-60 s in all brain

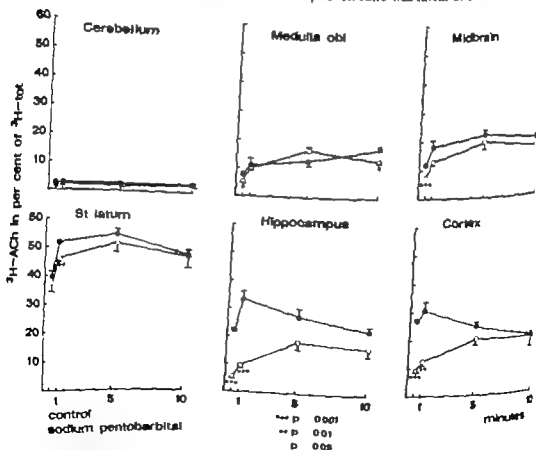


Fig. 1 Effect of sodium pentobarbital (60 mg/kg i.p.) on the biosynthesis of H-ACh in different brain regions. Each point represents the mean value of 3-6 expts. Vertical bars

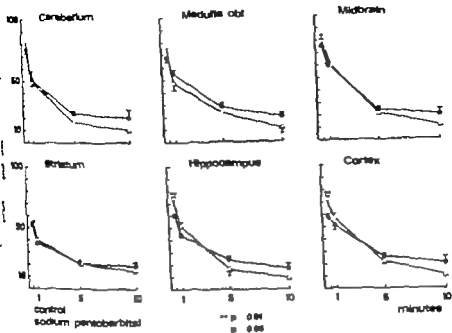


Fig. 2. Effect of sodium pentobarbital (80 mg/kg i.p.) on the concentration of untransformed $^3\text{H-Ch}$ in different brain regions. Each point represents the mean value of 3-6 expts. Vertical bars indicate S.E.

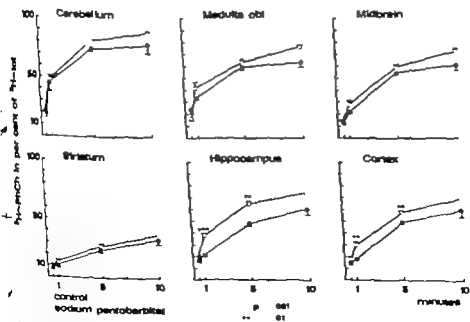


Fig. 3. Effect of sodium pentobarbital (80 mg/kg i.p.) on the biosynthesis of $^3\text{H-PbCh}$ in different brain regions. Each point represents the mean value of 3-6 expts. Vertical bars indicate S.E.

TABLE 1 Effect of sodium pentobarbital (60 mg/kg i.p.) on the steady state level of endogenous choline and acetylcholine (nmol g^{-1}) in discrete mouse brain regions. The animals were killed by microwave irradiation of the head (0.25 s \times 5 kW) 16 min after administration of sodium pentobarbital

Brain region	Choline		Acetylcholine	
	Control	Sodium pentobarbital	Control	Sodium pentobarbital
Cerebellum	32.8 ± 4.63 (10)	47.3 ± 3.49 (8)*	5.2 ± 1.05 (4)	5.7 ± 0.73 (4)
Medulla oblongata	43.8 ± 4.30 (8)	37.2 ± 4.89 (4)	29.1 ± 1.10 (4)	32.5 ± 2.38 (4)
Midbrain	33.8 ± 3.56 (9)	44.7 ± 7.80 (7)	29.0 ± 1.80 (6)	33.5 ± 1.27 (4)*
Striatum	38.2 ± 3.11 (10)	45.9 ± 6.30 (9)	76.8 ± 1.78 (3)	81.3 ± 2.62 (4)
Hippocampus	28.2 ± 3.18 (8)	32.2 ± 3.69 (8)	21.2 ± 2.32 (6)	39.8 ± 0.23 (2)*
Cortex	33.8 ± 3.34 (11)	39.0 ± 2.26 (9)	24.8 ± 1.44 (6)	43.1 ± 1.17 (4)*

Mean value (Mv) \pm S.E., (n) = number of expts.

$p < 0.001$

$p < 0.05$

In the cerebellum, medulla oblongata and striatum. The H-Ch concentration was 25% higher in the cortex and hippocampus than in the controls (Fig. 2).

The rate of H PhCh biosynthesis was increased at 1 min in the cortex and hippocampus (Fig. 3)

The time course of the change in the H ACh/H-Ch ratio was linear at 0-60 s in all brain

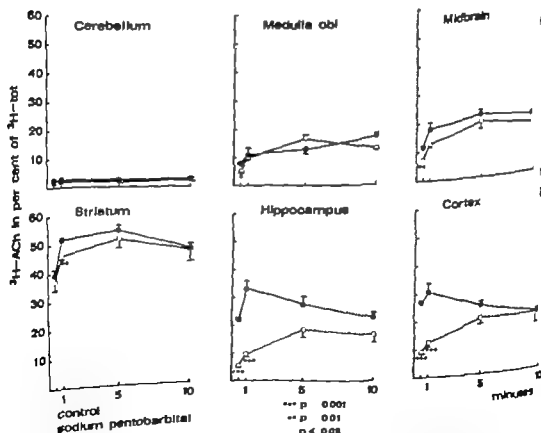


Fig. 1 Effect of sodium pentobarbital (60 mg/kg i.p.) on the biosynthesis of H-ACh in different brain regions. Each point represents the mean value of 3-6 expts. Vertical bars indicate S.E.

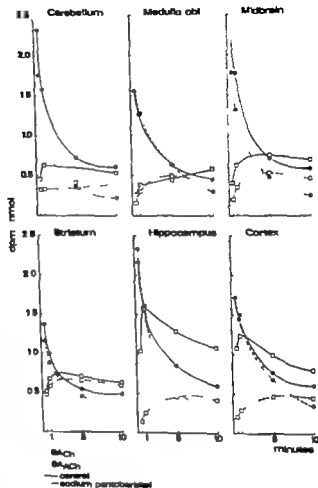


Fig. 3 Effect of sodium pentobarbital (60 mg/kg p) on the specific activities (SA) of ACh and Ch in different brain regions. SA are expressed as ^3H -ACh and ^3H -Ch as per cent of B-tar-mol endogenous ACh and Ch.

size taken to obtain complete inactivation of certain brain enzymes. In a previous paper Nordberg and Sundvall (1976) reported progressively higher values when three different methods of sacrifice were compared, *viz.* dislocation of the spine, whole body microwave irradiation (1.3 kW 7 s) and head irradiation (5 kW 0.25 s). The ACh in the striatum and cerebral cortex, in particular, appears to be extremely sensitive. Radioactive ACh synthesized by the brain from radioactive Ch appears to be more easily preserved than endogenous ACh, since maximum levels are obtained with both the whole body and head irradiation procedures, whereas dislocation of the spine gives low values, especially in the striatum (Nordberg and Sundvall 1976).

Following anaesthesia with sodium pentobarbital (60 mg/kg, i.p.), dislocation of the spine yields markedly increased steady state levels of ACh in the cortex, hippocampus and midbrain (130, 90 and 40%) but practically unchanged levels in the cerebellum, medulla oblongata and striatum (Nordberg and Sundvall 1975). The relative effects of sodium pentobarbital (per cent increase relative to control) are thus the same as when the animals are

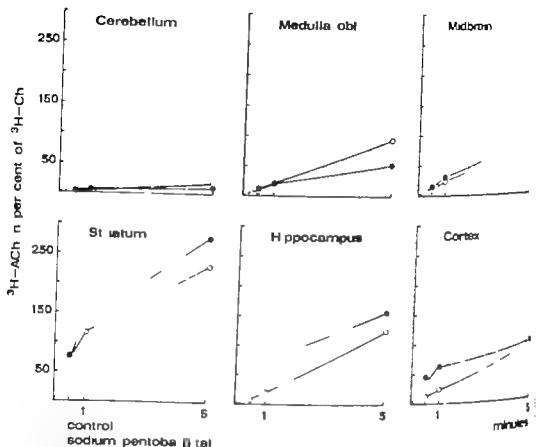


Fig. 4 Effect of sodium pentobarbital (60 mg/kg i.p.) on the $^3\text{H-ACh}/^3\text{H-Ch}$ ratio in different brain regions. Each point represents the mean value of 3-6 expts.

regions and was markedly decreased (by 60-80%) by sodium pentobarbital in the hippocampus and cerebral cortex (Fig. 4)

Effect on ACh turnover

The time courses of the changes in the specific radioactivities of ACh and Ch in the different brain regions are shown in Fig. 5 and Table II gives the apparent turnover calculated from the equation

$$\frac{\text{H-ACh} - \text{Ch nmoles}}{\text{H-Ch g min}} = \text{ACh nmol g}^{-1} \text{ min}^{-1}$$

As seen in the table, only in the cortex and hippocampus was the turnover markedly reduced after treatment with sodium pentobarbital.

Discussion

Microwave irradiation is used increasingly for killing small rodents when the steady state level of labile compounds such as ACh are to be studied. Generally higher values are found when this technique is used and it is obvious that the values obtained are related to the

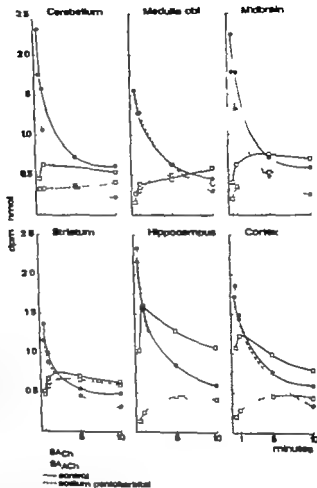


Fig. 5. Effect of sodium pentobarbital (60 mg/kg i.p.) on the specific acetylcholinesterase (SA) of Ch and Ch in different brain areas. SA are expressed as ^3H -Ch and ^3H -Ch in per cent of 8-hr control endogenous ACh of Ch.

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TABLE II Effect of sodium pentobarbital on the apparent turnover rate ($\text{nmol g}^{-1} \text{ min}^{-1}$) of acetylcholine in different brain regions.

Brain region	Control	Sodium pentobarbital
Cerebellum	(2)	(2)
Medulla oblongata	10	7
Midbrain	11	9
Striatum	55	59
Hippocampus	21	6
Cortex	27	10

killed by irradiation of the head (this paper). A different relative effect is obtained after whole body irradiation with the 1.3 kW irradiation source. In addition to the increase of ACh in the cortex, the hippocampus and midbrain, a marked increase is also found in the striatum and medulla oblongata (Nordberg and Sundwall 1975 unpublished data). The absolute ACh levels in nmol g^{-1} after administration of sodium pentobarbital, on the other hand, are exactly the same following the two types of microwave irradiation. It is the control values that differ. A possible explanation for these findings is that sodium pentobarbital stabilizes a very labile pool of endogenous ACh and/or that the anesthesia prevents or diminishes a heavy burst of ACh release to the synaptic cleft induced by the killing. This could explain the low values generally found after dislocation of the spine. This burst of ACh released into the synaptic cleft may be preserved by the rapid enzyme inactivation or it could be prohibited by the rapid tissue fixation produced by the microwave irradiation. The different results obtained with regard to endogenous and newly synthesized radioactive ACh may have a bearing on this, indicating that at least two pools of ACh are involved. In a recent paper Atweh and Kuhar (1976) found endogenous ACh in the hippocampus more labile to destruction than H ACh.

Also regarding the effect of sodium pentobarbital on endogenous ACh in the striatum, results differing from ours have been published. Modak *et al.* (1976) found an increase from 81 to 117 nmol g^{-1} (44%) in the striatum of mice killed by irradiation of the head for 0-300 s (6 kW) and Trabucchi *et al.* (1975) found an increase from 54 to 89 nmol g^{-1} (63%) in the striatum of rats following microwave irradiation of the head for 1.2 s (2.5 kW). The reason for these discrepancies is unknown.

For estimation of the ACh turnover an intravenous pulse injection of tracer amounts of radioactive Ch has been used (Schuborth *et al.* 1969). In the present study it has been ascertained that the ratio of radioactive ACh to Ch increases linearly with time in all brain regions, both in normal and sodium pentobarbital treated mice over the time interval 0 to 1.0 min (head irradiation). A precursor product relationship is also indicated by the fact that plots of the time course of the specific radioactivities (SA) of ACh and Ch intersect at the maximum SA of the product (ACh) in all brain regions except the cerebellum. This means that a basic criterion for a precursor product relationship would be fulfilled if in addition it were plausible that we have steady state, instant mixing and an open single compartment (Zilversmit *et al.* 1943). If this is assumed, it is possible to make a rough estimate of the turnover in the different regions. The highest turnover rate is found in the striatum (55 nmol

7). In the hippocampus and cortex the turnover is about half this value and in the oblongata and midbrain about one fifth. Sodium pentobarbital has a very specific effect on the turnover in the cortex and hippocampus. No other regions are affected. A similar effect has been found in the cortex and striatum by Trabucchi *et al.* (1975). The effect of sodium pentobarbital anesthesia on the sodium dependent high affinity uptake in synaptosomes from different brain regions have been demonstrated by *et al.* (1976). The anesthesia decreased uptake in the hippocampus and cortex but not effect on striatal synaptosomes. Regarding calculation of turnover rate, available data are rather imperfect and different values have been published. The figures given by *et al.* (1975 table 2) for the striatum ($18 \text{ nmol g}^{-1} \text{ min}^{-1}$) and cortex ($3 \text{ nmol g}^{-1} \text{ min}^{-1}$) are considerably lower than ours. They used a 6 min i.v. infusion of phosphorylcholine. In this case the difference may be due to the longer time interval (0–6 min) used for estimation of the rate of conversion of radioactive Ch into radioactive ACh. The time course of radioactive ACh/Ch ratio, obtained upon pulse injection is biphasic and the slope is usually less steep between 1 and 5 min. If this is also true following infusion of phosphorylcholine an explanation for the low turnover figures is found. At 5 min our values are $11 \text{ nmol g}^{-1} \text{ min}^{-1}$ for the cortex and striatum, respectively. Owing to the rapid turnover of ACh and the possibly preferential release of recently synthesized ACh with efficient degradation of the products of hydrolysis, the longer time points tend to give low values.

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Adrenergic and Cholinergic Nerves of the Human Urethra and Urinary Bladder. A Histochemical Study

By

A. EK, P. ALM, K.-E. ANDERSSON and C. G. A. PERSSON*

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Abstract

ALM, P., ANDERSSON, K.-E. and PERSSON, C. G. A. Adrenergic and cholinergic nerves of the human urethra and urinary bladder. A histochemical study. *Acta physiol. scand.* 1977 99 345-352.

The occurrence and distribution of adrenergic and acetylcholine esterase (AChE) positive nerves in the human urethra and urinary bladder were studied histochemically with the fluorochrome method of Falck and Hellery, and the copper-thiochrome method of Juelke and Friedewald. Both types of nerves were mainly confined to the layers of smooth muscle cells in the walls of the organs. In all parts of the urethra, there is a scanty supply of adrenergic nerves. Few adrenergic nerves were also found in the urinary bladder except in the trigone area, where they are abundant. AChE-positive nerves were uniformly and richly distributed in the urinary bladder. Throughout the urethra the distribution of AChE-positive nerve fibres was uniform, but the number was clearly less than in the urinary bladder. No intramurally located adrenergic or AChE-positive ganglion cells could be demonstrated.

The function of the parasympathetic nerves of the urinary bladder is well known from physiological and pharmacological investigations in man as well as in other species. The occurrence and distribution of acetylcholine esterase (AChE) positive nerves (supposed to be cholinergic) in the detrusor muscle have been described in various animals, but, surprisingly only very briefly and incompletely in man. Studies in normal man and in patients with various types of bladder dysfunction, performed *in vitro* and *in vivo* (Todd and Mack 1969, Nergårdh and Boreus 1972, Awad *et al.* 1974, Nergårdh and Gierup 1974, Calne *et al.* 1975, Ek *et al.* 1976, Sundin *et al.* 1976), have indicated an adrenergic influence on the bladder and bladder neck. Adrenergic nerve terminals, supposed to be the anatomical background for these effects, have been demonstrated in various bladder parts of different animals, but, so far only in the detrusor muscle and trigone area of man (Mobley *et al.* 1966, Sundin *et al.* 1976).

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nickelous method of Kuffler and Friederwald (1949), as modified by Holmstedt (1957), as used. Cryostat sections (about 10 μ m) are incubated for 10 h in acetylthiocholine iodide after preceding treatment with eserine (bis-monoisopropylamide phosphoryl fluoride, Swedish National Defence Research Inst.) for the inhibition of non-specific cholinesterase (Holmstedt 1957; Holmstedt and Sjöquist 1961) and/or with 24 C 51 (1-[5-iso-4-ethylthiomethyl] amino-2-naphthyl pentane-3-one-diuronide, Wellcome Res. Lab., Harlow) for the inhibition of specific AChE-activity (Bayliss and Tedrick 1956). After counterstaining in eosin, mounting was performed in Entellan (Merck).

Results

Adrenergic nerves

A considerable autofluorescence appeared in sections from all tissue specimens examined. It was mainly confined to the connective tissue in which bright yellowish fluorescent smooth muscle filaments appeared. These filaments had a uniform thickness along their whole extensions and ran with wave-like course more or less irregularly and dense to each other. The autofluorescence did not seemingly change qualitatively or quantitatively whether or not omitting the formaldehyde treatment step of the Falck and Hillarp technique for the very induction of the specific adrenergic nerve fluorescence. On the other hand, for differentiation, the adrenergic nerve terminals always demonstrated a distinct beaded appearance displaying a somewhat more greenish fluorescence. They were never seen when the formaldehyde treatment step was omitted. No evident difference in the number and distribution of fluorescent adrenergic nerves could be seen between irradiated and non-irradiated tissue specimens. The bundles of smooth muscle cells, forming the most basally located urethral structures, appeared distinctly dark against the autofluorescent connective tissue.

In both the male and female urethras, there was generally a very scarce supply of adrenergic nerves (see Fig. 2 a and b). Around the slender bundles of smooth muscle cells, very fine varicose adrenergic nerve terminals in a small number were seen. Mostly they ran longitudinally along the bundles of smooth muscle cells, but sometimes transversely running terminals could also be observed, connecting the longitudinally running terminals to a more or less distinct plexus of terminals around the bundles of smooth muscle cells. Adrenergic terminals were also found around some blood vessels, mostly occurring in the smooth muscle layers. The number and distribution of adrenergic nerve terminals was appreciably the same in male and female urethras and did not differ between the various urethral parts.

The urinary bladder also displayed a very scarce supply of adrenergic nerves. Except in the trigone area, terminals appeared in a very restricted number around bundles of smooth muscle cells in the lateral walls of the bladder in the same way as in the urethra (cf Fig. 2 a and b). Some blood vessels supplied with adrenergic terminals were also seen. In the trigone area, the number of adrenergic terminals around bundles of smooth muscle cells was much greater (see Fig. 2 c). The fluorescence picture was the same in male and female urinary bladders. No adrenergic ganglion cells could be seen, neither in the urinary bladder nor in the urethra.

AChE-positive nerves

In the urinary bladder of both males and females, the number of AChE-positive nerves was generally much greater than that of adrenergic nerves. Around the bundles of smooth muscle cells of the lateral walls of the bladder slender AChE-positive fibres occurring in a similar

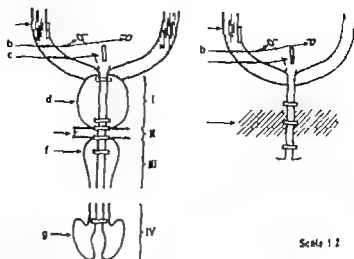


Fig. 1 Schematic picture of the male (left figure) and female (right figure) urethra and urinary bladder and some adjacent structures. Site of location for the examined pieces of bladder (I) and urethra (II) static urethra, II Membranous urethra, III Bulbous urethra, IV Penile urethra. a. Urinary bladder b. Orifices for ureters, c. Trigone area, d. Prostate e. Urogenital diaphragm, f. Corpus cavernosum, g. Glans penis.

During the last few years, several reports have stressed the clinical importance of adrenergic influence on the urethra. The therapeutic results of decreasing urethral resistance by α -adrenoceptor blocking agents in patients unable to empty their bladder for various reasons (Abel *et al* 1974, Khanna and Gonick 1975, Stockamp and Schreiter 1975, McNeil *et al* 1976, Whitfield *et al* 1976) seem to be well documented. In patients suffering urinary stress incontinence therapeutic effects of α -adrenoceptor stimulating agents (Dix and Taub 1975, Schreiter *et al* 1976, Stewart *et al* 1976) and β -adrenoceptor blocking (Gleason *et al* 1974) have also been reported.

Because of the scanty information on the innervation of the human urethra, and in order to better understand urethral mechanisms in further studies on urinary incontinence voiding disturbances in man, the present investigation was performed. It describes occurrence and distribution of adrenergic and AChE-positive nerves in the human urethra and the detrusor muscle and trigone area of the urinary bladder.

Material and Methods

In 5 men and 1 woman, who had received 4500 rad preoperatively and 3 men and 2 women, not operatively irradiated, total urethrectomy was performed because of locally wide spread infiltrating carcinoma of the bladder. Droperidol (Droloid, Leo, Sweden), fentanyl (Leptanal[®], Leo, S&L) and pancuron (Pavulon, Organon, Holland) were given for anaesthesia and preanaesthetic medication. Quickly after removal of the bladder tissue specimens were taken from macroscopically tumor free areas of the lateral walls and trigone area of the urinary bladder and from different parts of the urethra (Fig. 1). The specimens were then rapidly frozen in isopentane cooled by liquid nitrogen. For the fluorescence histochemical demonstration of adrenergic nerves according to the technique of Falck and Hall, pieces of tissue were freeze-dried, exposed to gaseous formaldehyde of standardized basicity and bedded in paraffin. Sections for fluorescence microscopy could then be made (for details, see Björklund *et al* 1972). For the histochemical demonstration of acetylcholine-esterase (AChE) activity the con-

uridine method of Kottle and Friederwald (1949), as modified by Holmsedt (1957), was used. Cryostat sections (about 10 μ m) were incubated for 10 h in acetylthiocholine iodide after preceding treatment with cupric (bis-monoisopropylamide phosphoryl fluoride, Swedish National Defence Research Inst.) the addition of non-specific cholinesterase (Holmsedt 1957 Holmsedt and Sjögquist 1961) and/or W 24 C 51 (1-5-bis 4-ethylthioethyl iminosulphonyl pentase-3-oxo-diboronide, Wellcome Res. Lab., U.K.) for the inhibition of specific AChE-activity (Bayliss and Todorick 1956). After counterstaining in eosin, mounting was performed in Eutellan (Merck).

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Adrenergic nerves

A considerable autofluorescence appeared in sections from all tissue specimens examined. It was mainly confined to the connective tissue in which bright yellowish fluorescent smooth muscle filaments appeared. These filaments had a uniform thickness along their whole extensions and ran in wave-like course more or less irregularly and dense to each other. The autofluorescence did not seemingly change qualitatively or quantitatively whether or not omitting the formaldehyde treatment step of the Falck and Hillarp technique for the very induction of the specific adrenergic nerve fluorescence. On the other hand, for differentiation, the adrenergic nerve terminals always demonstrated a distinct beaded appearance displaying a somewhat more greenish fluorescence. They were never seen when the formaldehyde treatment step was excluded. No evident difference in the number and distribution of fluorescent adrenergic nerves could be seen between irradiated and non-irradiated tissue specimens. The bundles of smooth muscle cells, forming the most basally located urethral structures, appeared distinctly dark against the autofluorescent connective tissue.

In both the male and female urethrae, there was generally a very scarce supply of adrenergic nerves (see Fig. 2 a and b). Around the slender bundles of smooth muscle cells, very few arcuate adrenergic nerve terminals in a small number were seen. Mostly they ran longitudinally along the bundles of smooth muscle cells, but sometimes transversely running terminals could also be observed, connecting the longitudinally running terminals to a more or less distinct plexus of terminals around the bundles of smooth muscle cells. Adrenergic terminals were also found around some blood vessels, mostly occurring in the smooth muscle layers. The number and distribution of adrenergic nerve terminals was appreciably the same in male and female urethrae and did not differ between the various urethral parts.

The urinary bladder also displayed a very scarce supply of adrenergic nerves. Except in the trigone area, terminals appeared in a very restricted number around bundles of smooth muscle cells in the lateral walls of the bladder in the same way as in the urethra (cf. Fig. 2 a and b). Some blood vessels supplied with adrenergic terminals were also seen. In the trigone area, the number of adrenergic terminals around bundles of smooth muscle cells was much greater (see Fig. 2 c). The fluorescence picture was the same in male and female urinary bladders. No adrenergic ganglion cells could be seen, neither in the urinary bladder nor in the urethra.

AChE-positive nerves

In the urinary bladder of both males and females, the number of AChE-positive nerves was generally much greater than that of adrenergic nerves. Around the bundles of smooth muscle cells of the lateral walls of the bladder slender AChE-positive fibres occurring in a similar

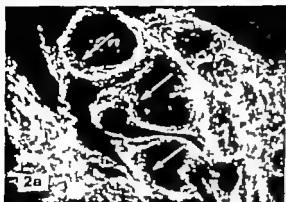


Fig. 2 a. Urethra. The bundles of smooth muscle cells are transversely sectioned and seen in the figure as a dark area surrounded by close encircling adrenergic nerve terminals (g). Fluorescence micrograph, 175

Fig. 2 b. Urethra. Longitudinal section of a bundle of smooth muscle cells seen in the figure as a dark area surrounded by bright autofluorescence from fibrous tissue. Fine varicose adrenergic nerve terminals (g) are seen in the bundle of smooth muscle cells along its longitudinal direction. Fluorescence micrograph, 175

Fig. 2 c. Urinary bladder trigone area. Numerous adrenergic nerve terminals with high fluorescence intensity and a somewhat irregular course occur here (g). Fluorescence micrograph, 175

number and with the same distribution in all regions of the bladder formed net-like plexuses (see Fig. 3 a). The muscle bundles had a more dense supply of AChE positive nerves than of fluorescent adrenergic nerve terminals. In the basal layers of smooth muscle cells, large AChE positive nerve trunks appeared. In some of the blood vessels of these layers, AChE positive nerve fibres were found to end in the vascular adventitia.

Both the male and female urethra demonstrated a similar pattern of distribution of AChE-activity as here described for the urinary bladder. However, in the urethra the occurrence of AChE positive nerve fibres around the bundles of smooth muscle cells was not as dense as in the bladder. It seemed to be more dense than the corresponding occurrence of fluorescent adrenergic nerve terminals, see Fig. 3 b. The distribution and number of AChE positive nerve fibres were not found to differ between various parts of the urethra. No AChE positive ganglion cells were observed in the lateral walls or in the trigone area of the urinary bladder or in the urethra. The number and distribution of AChE-positive nerves were similar in irradiated and non-irradiated tissue specimens.

Discussion

For the histochemical demonstration of adrenergic nerves, the fluorescence method of Falck and Hillarp is widely used. Its very prominent sensitivity and specificity is well known and



Fig. 3. Urinary bladder. Rich distribution of AChE-positive nerves in the somewhat dark counterstained areas separating bundles of smooth muscle cells. Light micrograph, 110.

Fig. 3b. Urethra. Compared with Fig. 3 there is less AChE-positive nerves in relation to the bundles of smooth muscle cells. Light micrograph, 110.

has been thoroughly discussed (Björklund *et al.* 1972). To demonstrate AChE-positive nerves, the copper thiocholine technique of Koelle and Friedenwald is at present one of the most widely used methods. However, it is less sensitive and specific than the Falck and Hillarp technique for adrenergic nerves. To get the optimum and most specific demonstration of AChE-positive nervous structures, various methodological conditions should be fulfilled and always tested for each species and tissue studied (*cf.* Ehinger 1966). This was done in pilot experiments in this study.

The present investigation showed that there was a very scarce supply of adrenergic nerve terminals in the lateral part of the bladder compared to the rich supply in the trigone area. On the other hand, there was a rich supply of AChE-positive fibres, which was appreciably the same in the investigated bladder regions. This was valid both in parts displaying very few adrenergic nerves and in the trigone area. In view of this, it seems reasonable to assume that the copper thiocholine method almost exclusively demonstrates AChE-positive nerves and not, or to a very small extent, adrenergic terminals.

The number and general distribution of adrenergic nerves in the human urinary bladder reported here and recently by Sundin *et al.* (1976), seem to agree with findings in other species such as cat, dog, rabbit, rat, and guinea-pig (Hamburger and Norberg 1965, El-Badawi and Schenk 1966, Owman and Sjöberg 1972, Wakado and Kirpekar 1972, Raczer *et al.* 1973, 1976, Sundin and Dahlström 1973, Dixon and Gosling 1974, Aln and Elmér 1975). Also the uniform, rich supply of AChE-positive fibres in the human urinary bladder is only very briefly and incompletely described before (Mobley *et al.* 1966, Nyo 1969, Faerman

et al 1971) is similar to findings described in cat, dog, rabbit, rat, and guinea-pig (El-Badawi and Schenk 1966, Raezer *et al.* 1973, 1976, Dixon and Gosling 1974, Alm *et al.* 1975). However, the present work demonstrates a very sparse supply of adrenergic nerves in all parts of the human urethra. This is in contrast to observations in other species such as cat, dog, guinea-pig, and rat, demonstrating a rich supply of adrenergic nerve terminals, somewhat denser in the proximal part of the organ (Owman *et al.* 1971, Owman and Sjöberg 1972, Sundin and Dahlström 1973, Benson *et al.* 1976, Sjöberg *et al.* 1976). Furthermore, the present study describes a rather rich supply of AChE positive nerves in all parts of the human urethra. This is interesting, as there seem to be no earlier studies in any species describing AChE-positive nerves in the urethra. Thus, the occurrence and distribution of adrenergic and AChE positive nerves of the human urinary bladder are in accordance with findings in several other species. On the other hand, the human urethra seems to differ in these respects from other species.

The uniform although scarce occurrence of adrenergic nerves throughout the human urethra suggests a constant relationship between nerve terminals and muscle cells. This finding also suggests that there are no functional differences between the effects exerted by the adrenergic nerves in the different parts of the organ. This view is supported by studies showing a uniform contractile response of the smooth muscle in all parts of the human urethra induced by α -adrenoceptor stimulating drugs (Ek *et al.* 1976).

The functional importance of the AChE positive nerves of the urethra is unknown. Studies *in vitro* on the isolated human urethra showed that exogenous acetylcholine, even when administered in high concentrations, had insignificant effects (Ek *et al.* 1976). This seems somewhat contradictory to the occurrence of AChE positive nerves forming net-like plexus around the bundles of smooth muscle cells. However, one explanation might be that cholinergic nerves of the human urethra do not directly exert physiological influence on the smooth muscle cells. Hypothetically they could form synapses with the adrenergic nerves and in some way interact with these in the contractile response. Interaction between adrenergic and cholinergic nerves was originally suggested by Burn and Rand (1959, 1965) and has later been extensively studied (see Spörriog 1970).

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Lower Body Negative Pressure and Effects of Autonomic Heart Blockade on Cardiovascular Responses

By

H. BJURSTEDT, G. ROSENHAMER and G. TYDÉN

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Abstract

BJURSTEDT H, G. ROSENHAMER and G. TYDÉN. Lower body negative pressure and effects of autonomic heart blockade on cardiovascular responses. *Acta physiol. scand.* 1977 99 353-360.

Heart rate, arterial pressure and cardiac output were recorded in eight healthy male volunteers during exposure to 80 mmHg of lower body negative pressure (LBNP) in the supine position before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade of the heart as induced by the administration of propranolol 0.25 mg/kg b.wt. and atropine 0.04 mg/kg b.wt. After propranolol, heart rate response to LBNP averaged 44% of that observed without blockade indicating that LBNP-induced cardiovascular responses of both sympathetic and parasympathetic origin. Tolerance to LBNP was reduced by beta-adrenergic blockade, since the decrease in mean arterial pressure during LBNP was augmented by such blockade. Although the addition of atropine markedly elevated mean arterial pressure and cardiac output in the control situation, tolerance to LBNP was not enhanced by this drug as judged from the arterial pressure response. Post LBNP overshoot in mean arterial pressure was strikingly augmented by combined cardiac effector blockade and was in part due to a lagging elevation of total peripheral resistance. Cardiac output remained decreased for more than 110 s after release of LBNP.

The arterial pressure response to a threefold increase of the gravitational force in the head-foot direction, associated with massive hypotension at head level and drainage of blood into the dependent part of the body is not affected by beta-adrenergic blockade but is reduced after combined beta-adrenergic and parasympathetic blockade (Bjurstedt, Rosenhamer and Tydén 1974, 1976). However the pressure response to a local decrease in the carotid sinus transmural pressure is not affected by autonomic heart blockade (Bjurstedt, Rosenhamer and Tydén 1975). It seemed of interest therefore to study the effects of autonomic heart blockade on the circulatory adaptation to lower body negative pressure (LBNP), since this maneuver may produce considerable pooling of blood in dependent veins with little if any effect on the arterial blood pressure at head level (for review see Wolzbach, Bergman and Naegele 1974). Accordingly we have investigated, in human subjects, the cardiovascular effects of 80 mmHg of LBNP before and during beta-adrenergic and combined beta-adrenergic and parasympathetic blockade as induced by the intravenous administration of propranolol and atropine.

Material and Methods

8 healthy well trained male volunteers served as subjects. Individual and dimensional data are given in Table I.

The device used to produce LBNP consisted of a wooden box 130 × 50 × 36 cm with an elliptical opening at one end. A rubber seal was provided at the opening which fitted hermetically around the neck at level of the iliac creas. The subject rested supine in the box straddling a narrow padded saddle which provided support during the suction exposures. The box was connected via a pressure regulator to a remotely located prevacuated low pressure chamber which made possible the application of graded negative pressure. The time to reach the desired negative pressure was kept at about 30 seconds. A strain-gauge manometer was used for monitoring the pressure in the box. The temperature inside the box was monitored continuously and was kept within $\pm 0.5^{\circ}\text{C}$.

Intraarterial pressure at heart level and heart rate were measured according to methods described elsewhere (Bjurstedt, Rosenhamer and Tydén 1974). Dichromatic carotid densitometry was used for determination of cardiac output. This method, described Reed and Wood (1967), has shown satisfactory agreement with results obtained with arterial cuvette densitometry (Reed and Wood 1967; Schneider, Chazadeh and Möller 1967; Wallgren 1975). The dichromatic carotid (Waters XE 30a) was attached to the ear after rubbing the pinna for 1 min with rubrifacient ointment (Transvasin®) and was secured to the ear with adhesive tape. The ear and carotid were then covered with green cloth to prevent influence of ambient light on the photocell. Calibration was achieved according to the end-tail method (Beard and Wood 1967).

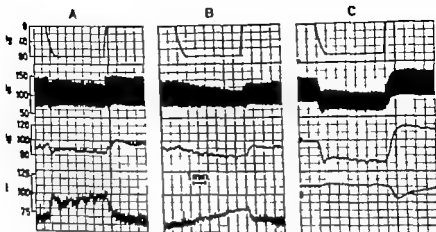
Design of experiment. Prior to the actual experiment subjects had been made familiar with the subjective sensations associated with LBNP. The experiment commenced with a 15 min period of rest in the supine position in the box followed by a 4 min exposure to 80 mmHg LBNP. Beta-adrenergic blockade was then induced by the intravenous administration of 0.25 mg/kg of propranolol over a 3 min period whereupon the 15 min period of rest and the 4 min exposure of 80 mmHg were repeated. Atropine (0.04 mg/kg) was then injected and the 15 min period of rest and 4 min period of LBNP were again repeated. Determinations of cardiac output were made during the last 3 min of each 15 min rest period and 3 consecutive determinations recorded during LBNP the first determination being made 20 s after attainment of 80 mmHg LBNP, second after 1 min 50 s and the third after 3 min 20 s. Following the cessation of LBNP another two determinations of cardiac output were performed, the first after 70 s and the second after 1 min 50 s. Heart rate, arterial pressure and mean arterial pressure derived electronically were recorded continuously throughout the experiment.

Calculations. Heart rate and mean arterial pressure were averaged during the inscription of each indicator dilution curve. In the resting condition the mean values of the two cardiac output determinations and the corresponding values of heart rate and mean arterial pressure were calculated. Total peripheral resistance was calculated as mean arterial pressure (mmHg) divided by cardiac output (ml/s). The statistical significance of differences between mean values were evaluated by applying the *t*-test to the individual differences (cf. Fischer 1948).

TABLE I. Functional and dimensional data.

Subject	Age, years	Weight, kg	Height, cm	BSA, m^2	HR	MAP
JK	37	70	180	1.88	59	90
JT	35	74	181	1.94	62	88
PH	34	88	184	2.10	47	9
MK	21	87	186	2.11	55	93
JP	27	63	173	1.75	54	88
LÖ	31	65	174	1.78	75	100
AA	26	85	190	1	55	81
FA	28	73	183	1.94	65	91

BSA = Body surface area from nomogram of Dubois and Dubois (1916). HR = Heart rate in beats/min after 15 min of supine rest. MAP = Mean arterial pressure in mmHg at heart level after 15 min of supine rest.



Figures of original recording showing time-dependent effects of LBNP (-80 mmHg), on arterial pressure in the radial artery (AP), mean arterial pressure (MAP) obtained electronically from AP and heart rate (A) and after beta-adrenergic (B) and combined beta-adrenergic and parasympathetic (C) blockade.

Results

Figure 1 shows an example of the circulatory changes produced by the application of -80 mmHg LBNP before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade. Mean values of the observed responses are given in Table II-IV. Although all of the subjects showed a progressive fall in mean arterial pressure during LBNP, none displayed any symptoms of presyncopal nature.

Responses to -80 mmHg of LBNP before blockade. With the application of negative pressure to the lower limbs, stroke volume showed a progressive decrease (-52% after 30 s, -60% after 3 min 20 s), while heart rate gradually increased (+41% and 66%, respectively). Cardiac output, therefore, decreased initially by 33% but did not show any further reduction. Mean arterial pressure decreased towards the end of the LBNP period (-9% after 3 min 20 s), due to the fall of the total peripheral resistance to remain at the same high level throughout this period (-30% after 20 s, -40% after 3 min 20 s).

After release of the negative pressure mean arterial pressure showed a slight, insignificant, rebound accompanied by a moderate slowing of the heart rate (-8%, $p < 0.05$). Cardiac output remained decreased 20 s after cessation of LBNP (-11%, $p < 0.001$) as compared to its value before negative pressure, whereas total peripheral resistance was increased 18% after 20 s.

Responses to -80 mmHg of LBNP after propranolol. After propranolol LBNP reduced mean arterial pressure by 12% after 3 min 20 s, the absolute value being significantly lower than before blockade (78 mmHg and 83 mmHg, respectively, $p < 0.05$). The LBNP-induced deceleration was markedly reduced (+16% after 20 s, +39% after 3 min 20 s) while reduction in stroke volume was much less evident than before propranolol. Cardiac output was therefore reduced only by 18% after 20 s and by 24% after 3 min 20 s of LBNP.

TABLE II Responses to 80 mmHg LBNP without blockade.

	Before LBNP	During LBNP			After LBNP	
		20 s	1 min 50 s	3 min 20 s	20 s	1 min 50 s
MAP	91±	89±2	86±3	83±3	94±3	93±
diff		-2±1	-5±2	-8±2	+3±3	+2±2
HR	59±3	83±4	92±4	98±5	60±5	54±3
diff		+24±4	+33±4	+39±5	+1±2	5±1
Q	8.1±0.4	5.4±0.4	5.4±0.4	5.4±0.4	7.2±0.3	7.7±0.3
diff		-2.7±0.1	-2.7±0.1	-2.7±0.1	-0.9±0.1	0.4±0.4
TPR	0.68±0.03	1.02±0.07	0.99±0.06	0.95±0.06	0.80±0.05	0.75±0.04
diff		+0.34±0.04	+0.31±0.03	+0.27±0.04	+0.12±0.03	+0.07±0.05
SV	140±9	67±6	58±4	56±4	124±9	147±13
diff		-73±6	-82±6	-84±7	-16±4	+7±9

MAP=mean arterial pressure, mmHg. HR=heart rate, beats/min. Q=cardiac output, l/min. TPR peripheral resistance, mmHg s/ml. SV=stroke volume, ml. denotes statistical difference from control value before LBNP -p 0.05 -p 0.01 -p 0.001

TABLE III Responses to 80 mmHg LBNP after propranolol.

	Before LBNP	During LBNP			After LBNP	
		20 s	1 min 50 s	3 min 20 s	20 s	1 min 50 s
MAP	89±2	85±2	81±1	78±2	90±3	89±2
diff		-4±1	-8±2	-11±2	+1±1	0±1
HR	49±2	57±3	66±2	68±2	49±3	48±3
diff		+8±1	+17±2	+19±2	0±1	-1±1
Q	5.5±0.3	4.5±0.3	4.2±0.2	4.0±0.3	5.0±0.3	5.6±0.4
diff		-1.0±0.3	-1.3±0.2	-1.3±0.2	0.5±0.2	+0.1±0.2
TPR	1.00±0.07	1.17±0.10	1.20±0.08	1.16±0.4	1.11±0.10	1.00±0.08
diff		+0.17±0.06	+0.20±0.04	+0.16±0.07	+0.11±0.05	0.00±0.03
SV	116±10	81±7	64±5	63±6	107±11	120±14
diff		-35±6	-52±6	-53±6	-9±6	+4±8

Abbreviations as in Table II

TABLE IV Responses to 80 mmHg LBNP after propranolol and atropine.

	Before LBNP	During LBNP			After LBNP	
		20 s	1 min 50 s	3 min 20 s	20 s	1 min 50 s
MAP	100±3	85±2	84±2	79±2	111±5	117±4
diff		-15±3	-16±3	-21±3	+11±3	17±3
HR	91±4	95±3	96±3	96±3	82±4	88±4
diff		+4±2	+5±3	+5±2	9±1	3±1
Q	7.6±0.6	4.0±0.3	4.0±0.3	4.1±0.3	6.5±0.5	7.3±0.5
diff		-3.6±0.4	-3.6±0.4	-3.5±0.5	1.1±0.3	0.3±0.2
TPR	0.82±0.07	1.32±0.10	1.31±0.10	1.20±0.11	1.08±0.11	0.98±0.07
diff		+0.50±0.06	+0.49±0.05	+0.38±0.08	+0.25±0.08	0.16±0.08
SV	86±8	43±4	42±4	43±4	81±9	86±7
diff		-43±5	-44±5	43±6	-5±2	0±

Abbreviations as in Table II

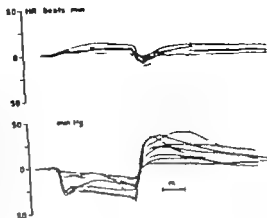


Fig. 2. Individual responses in heart rate (HR) and mean arterial pressure (MAP) during and after 4 min of LBNP after combined beta-adrenergic blockade, withdrawn from original recordings (all pre-LBNP values set to zero). Note residual changes in heart rate upon withdrawal of prepropranolol and atropine, also remarkably strong and long lasting post-LBNP overshoots in mean arterial pressure.

in absolute value, however being significantly less than before blockade. The maximal increase in calculated total peripheral resistance was 20%.

When pressure in the box was returned to ambient, cardiac output and total peripheral resistance remained temporarily somewhat decreased and increased, respectively as compared to pre-LBNP values whereas mean arterial pressure and heart rate rapidly returned to normal.

Responses to 80 mmHg or LBNP after propranolol and atropine. After combined beta-adrenergic and parasympathetic blockade LBNP produced a marked fall in mean arterial pressure (15% after 20 s and -21% after 3 min 20 s). However the absolute value after 3 min 20 s was not significantly lower than with propranolol alone. Heart rate increased by 5% ($p < 0.05$). Stroke volume decreased by 50% within 20 s and did not show any further reduction, its absolute value being significantly lower than during LBNP with no blockade or with propranolol alone. Cardiac output was reduced by no less than 47% already after 20 s. Calculated total peripheral resistance therefore showed a marked increase (61% after 20 s, 46% after 3 min 20 s).

In the recovery period after LBNP following autonomic heart blockade, mean arterial pressure showed marked and prolonged overshoots in some individuals (Fig. 2). For the group, the overshoot was 11% after 20 s and 17% after 1 min 50 s. The overshoot was associated with a clearcut slowing of the heart rate. As was the case with no drug and with propranolol alone cardiac output remained decreased for at least 1 min 50 s. The return of total peripheral resistance towards normal was remarkably sluggish, this variable still being elevated by 20% after 1 min 50 s as compared to its pre-LBNP value.

Discussion

Lower body negative pressure is a powerful tool for altering cardiovascular dynamics and has been shown to produce many of the same changes in cardiac output, heart rate and arterial pressure that occur with upright tilting. Indeed, most previous investigators of the effects of LBNP have been interested in pressure ranges that produce physiological changes

comparable to those observed during orthostasis and 70° head-up tilt, *i.e.* 40–50 mmHg (for review see Wolthuis, Bergman and Nicogossian 1974). For this reason, information is scarce as to the effects of LBNP of greater magnitude. Furthermore, it seems that only a few studies have included measurements of cardiac output, intraarterial pressure and heart rate during LBNP (Stevens and Lamb 1965, Murray *et al.* 1968, Rowell *et al.* 1972).

In the control situation in the present experiment LBNP produced a drastic reduction in stroke volume (52% after 20 s, 60% after 3 min 20 s) accompanied by a clearcut increase in heart rate (41% after 20 s, 66% after 3 min 20 s). These findings are in agreement with those of Stevens and Lamb (1965) who used the same level of negative pressure. Stevens and Lamb extended the LBNP periods until signs of presyncope occurred and found that the subjects could be divided into symptomatics and asymptomatics *i.e.* those who experienced presyncopal symptoms within 5 min and those who did not. It was noted that asymptomatic subjects did not show any decrease in mean arterial pressure during 5–3 min of exposure to 80 mmHg while symptomatics did. Although the present LBNP exposures were not extended beyond 4 min they support the findings of Stevens and Lamb since it was found that not all subjects tolerated the exposure well while others showed a slight but continuous fall in mean arterial pressure during LBNP. In the latter case the fall in blood pressure was due to failure to maintain total peripheral resistance throughout the LBNP period while cardiac output was well maintained.

After propranolol tolerance to LBNP was reduced as judged from the blood pressure response *i.e.* mean arterial pressure was significantly lower after 3 min 20 s as compared to the situation before blockade. This can be explained by the negative chronotropic effect of propranolol and the resulting reduction in cardiac output. Although there was a relatively potent vasoconstrictor response to LBNP after propranolol it was not sufficient to maintain arterial pressure at the same level as before blockade. This finding is notable since the exaggerated curtailment of cardiac output occurring during head-up tilt (Sannerstedt, Julius and Conway 1970, Loeppky 1975) or exposure to increased gravitational force (Burns *et al.* 1974) after propranolol is well compensated by a concomitant rise in peripheral resistance. In the latter cases the carotid sinus hypotension associated with head-up tilt or increased gravitational force may initiate a stronger vasoconstrictor reflex than does LBNP.

After propranolol, heart rate response to LBNP was only 48% of that observed before blockade, the absolute values after 3 min 20 s being 68 beats/min and 91 beats/min, respectively. This finding implies that the heart rate response to LBNP is of both sympathetic and parasympathetic origin. It also seems probable that vagal withdrawal was far from maximal during LBNP since heart rate at the end of the LBNP period after propranolol (68 beats/min) was much lower than that obtaining after propranolol and atropine (91 beats/min at rest).

With combined cardiac effector blockade heart rate, cardiac output and mean arterial pressure increased considerably, these findings being concordant with other experiments concerning autonomic heart blockade in the recumbent position (Julius *et al.* 1971, Nordenfält 1971, Ellis and Julius 1973, Korner *et al.* 1973, Bjurstedt *et al.* 1975). However, following application of LBNP there was a drastic reduction in cardiac output and mean arterial pressure to the same levels as obtained during LBNP with propranolol only. These findings favor

cept that atropine does not enhance the tolerance to hydrostatically induced blood volume placement as brought about by LBNP (Murray and Shropshire 1970), head-up tilt table, Leonard and Warren 1957) or increased gravitational force (Bjurstedt *et al.* 1976). At the termination of negative pressure before blockade there was a small overshoot in arterial pressure which was found to be due to a persisting increase in total peripheral resistance, cardiac output remaining temporarily suppressed. This is in accordance with the drop of a sustained increase in vasoconstrictor tone after the Valsalva maneuver (*cf* Sharpey-Schafer 1963), this maneuver producing similar changes in venous return as LBNP. There is also a direct correlation between the rise in peripheral resistance during the Valsalva maneuver and the magnitude of the ensuing overshoot in mean arterial pressure (Sharpey-Schafer 1963). The finding that the LBNP-induced increase in total peripheral resistance was widely enhanced by combined blockade therefore at least partly explains the remarkable overshoot in mean arterial pressure after propranolol and atropine (see Fig. 2). A contributing factor may have been blockade of beta receptor mediated vasodilatation (*cf* *et al.* 1966, Mahon 1966).

Concomitantly with the overshoot in mean arterial pressure a slowing of the heart was noted, the magnitude of which was directly related to the intensity of the overshoot. This slowing of the heart rate is probably the result of reflexes originating in arterial stretch receptors responding to the increase in arterial pressure. Involvement of low pressure receptors is likely since the effect of increased venous return on intrathoracic low pressure receptors is a speeding rather than a slowing of the heart rate (Bainbridge 1915 Linden 1972). The fact that the post-LBNP bradycardia was not abolished by the combined administration of atropine and propranolol is notable since no slowing of the heart rate occurs in the denervated heart following the Valsalva maneuver or a change from erect to recumbent posture (Beck, Bernard and Schrire 1969 Simson *et al.* 1970). It can be assumed therefore that the overshoot in arterial pressure exerted a reflex influence on the cardioinhibitory center of such a magnitude as to make the cardiac effector blockade incomplete even though the doses of atropine and propranolol used in the present experiments should normally produce a almost complete sympathetic and parasympathetic isolation of the heart (*cf* Jose 1966).

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Abstract

BRISMAR, T. *Slow action of Ca on myelinated nerve fibres of Xenopus laevis*. Acta physiol. scand. 1977 99 361-367

Myelinated nerve fibres were isolated and the nodal currents were recorded with potential clamp steps. Rapid solution changes were performed by use of a recording chamber so shaped that it had small dead space. A volume ten times larger than the dead space flowed past the node within about 20 ms. Half-time of the change in Na current (I_{Na}) amplitude associated with changes between solutions of different [Ca] was 180-150 ms, whereas the half-time of the change in I_{Na} associated with changes between solutions of different [Ca] was 200-300 ms. The external [Ca] affects I_{Na} through its action on the Na permeability properties of the nodal membrane. The difference in rate of effect between changes in [Na] and [Ca] is also noticeable. Less systematic changes were performed. These findings are discussed with regard to the properties of the nodal gap.

Key words: Node of Ranvier, unmyelinated axons, calcium, Na-permeability

The action of Ca on the ionic permeability in nerve has been studied in several potential clamp investigations (Frankenhaeuser and Hodgkin 1957, Hille 1968, Brismar and Frankenhaeuser 1972, 1975, Brismar 1973, Vogel 1974). At high [Ca] in the external solution larger relative potential changes are required to turn on the Na-permeability. The effect of an increase in [Ca] is then, with regard to the permeability properties, essentially equivalent to a negative potential change. However, the maximum Na-permeability at large positive potential steps is slightly smaller in high [Ca] than in low [Ca]. The effect of Ca is reversible and complete within the time usually required for the solution change (about 5 ms in the experiments on myelinated nerve fibres).

Several investigators have concluded that the action of Ca might be due to neutralization of negative charges assumed to be fixed onto the external surface of the membrane (Moxley and Naumov 1970, Brismar 1973, D'Arrigo 1973, 1974, 1975, Ehrenstein and Gilbert 1973, Hartz and Ulbricht 1973, Vogel 1974, Schauf 1975). Ca would change the electric field within the membrane and consequently the relation between permeability and potential. Neutralization of the fixed charges is assumed to be caused through screening by the cations in the external solution according to the theory of Gouy and Chapman for electric double layers (cf. Grahame 1947).

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Myelinated nerve fibres were isolated and the nodal currents were recorded with potential clamp technique. Rapid solution changes were performed by use of a recording chamber so shaped that it had a small dead space. A volume ten times larger than the dead space flowed past the node. When about 20 ms after the change in Na current (I_{Na}) amplitude associated with changes between solutions of different $[Na]$ was 180-190 ms, whereas the half-time of the changes in I_{Na} associated with changes between solutions of different $[Ca]$ was 200-300 ms. The external $[Ca]$ effects I_{Na} through its action on the Na permeability properties of the nodal membrane. The difference in rate of effect between changes in $[Na]$ and $[Ca]$ was noticeable when semiautomatic changes were performed. These findings are discussed with regard to properties of the nodal gap.

Key words: Node of Ranvier; mucopolysaccharides; calcium; Na-permeability.

The action of Ca on the ionic permeability in nerve has been studied in several potential clamp investigations (Frankenhaeuser and Hodgkin 1957, Hille 1968, Brismar and Frankenhaeuser 1972, 1975, Brismar 1973, Vogel 1974). At high $[Ca]$ in the external solution larger positive potential changes are required to turn on the Na-permeability. The effect of an increase in $[Ca]$ is thus, with regard to the permeability properties, essentially equivalent to a negative potential change. However, the maximum Na-permeability at large positive potential steps is slightly smaller in high $[Ca]$ than in low $[Ca]$. The effect of Ca is reversible and complete within the time usually required for the solution change (about 5 s in the experiments on myelinated nerve fibres).

Several investigators have concluded that the action of Ca might be due to a neutralization of negative charges assumed to be fixed onto the external surface of the membrane (Mozeyers and Neumov 1970, Brismar 1973, D'Arrigo 1973, 1974, 1975, Ehrenstein and Gilbert 1973, Hartz and Ulbricht 1973, Vogel 1974, Schauf 1975). Ca would change the electric field within the membrane and consequently the relation between permeability and potential. Neutralization of the fixed charges is assumed to be caused through screening by the cations in the external solution according to the theory of Gouy and Chapman for electric double layers (cf. Grahame 1947).

The present investigation was performed in order to find out whether the action of Ca is slow or rapid at very rapid changes between external solutions of different [Ca]_o. Techniques for rapid switches between solutions with different compositions have been used for the isolated myelinated nerve fibre. Thus Stämpfli (1958) has used a small stop cock combined with a small dead space in order to obtain rapid switches. Vierhaus and Ulbricht (1971) have measured the rate of such rapid switches through observations of the changes in Na currents during switches between solutions with different [Na]_o. They found on fibres from *Rana esculenta* that the change in external [Na] followed an exponential time course with a time constant of 30–50 ms. In order to press the rate for such a solution switch still further the present experiments on *Xenopus* fibres were performed with a dead space minimized to 0.02 mm³. In spite of that a 50% complete solution switch required 100–150 ms, as judged from the changes in *I*_{Na} (Na-current) at a rapid switch between two solutions with different [Na]_o. The half time for the effect of rapid changes in [Ca] was twice as long or 200–300 ms. The difference in rate of action between Na and Ca will be referred to the ultrastructure of the node region and the properties of the node gap substance.

Methods

Potential clamp experiments were performed on single myelinated nerve fibres isolated from the sciatic nerve of the frog (*Xenopus laevis*). The potential clamp technique was essentially similar to the one described by Dodge and Frankenhaeuser (1958). A rapid switch from one solution to another one was obtained by letting an amount of new solution, which was usually held together by surface tension as isolated from the pool containing the node under observation suddenly fuse with the pool and flow past the node. The dead space in the pool upstream the node was kept at a practical minimum of about 0.02 mm³. The solution flow was then kept unidirectional past the node in order to prevent back flow of the old solution. The time for contact made between the two solutions was monitored electrically. The rate of change in ionic concentration at the axon membrane surface was measured from the change in the peak amplitude of the inward membrane current during short test pulses repeated every 50 ms. The half time of the solution changes was estimated from records of membrane current in the individual node tested. It's a half-and-half mixture of the basic solutions. The membrane was polarized to -120 mV between the test pulses. The polarization removed the Na⁺ activation caused by the test pulses. The composition of the test solutions is described in Table I. All solutions were buffered with 2.5 mM Tris to pH 7.4 ± 25°C.

Results

Fig. 1A is a plot of the peak membrane current (mainly carried by Na⁺) associated with short lasting test steps to -30 mV with a conditioning membrane polarization to -120 mV. The [Ca] was held at 0.27 mM throughout while the [Na] was 25 mM before the solution switch and 115 mM after the switch. The Na permeability (*P*_{Na}) was nearly fully turned on.

TABLE I. Composition of test solutions (mM)

No	1	2	3	4	5
NaCl	115	115	25	25	11
CaCl ₂	14.8	0.27	14.8	0.27	2.0
KCl	—	—	90	90	2.5
Tris	2.5	2.5	2.5	2.5	2.5

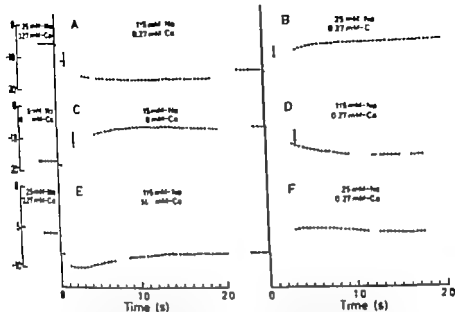


Fig. 1. Changes in total membrane current associated with rapid changes in ionic composition of external saline (indicated). Repeated positive potential pulses to -30 mV from continuous polarization to -120 mV increase test pulses. Duration of test pulses 5 ms. Repetition rate 23 per s. Repeating of solution changes indicated by the vertical lines and by arrows: the half-time of the solution changes. Temperature 9°C .

the test pulse with the fibre in these solutions. The plot ought therefore to indicate the rate of change of $[\text{Na}]$ immediately outside the membrane. Thus the concentration change was half way complete in about 150 ms. The switch back to low $[\text{Na}]$ was similarly 50% complete within about 150 ms (Fig. 1B).

Rapid changes in $[\text{Ca}]$ were performed in a similar way. The potential of the test pulse was then adjusted to the potential region where P_{Na} was most affected by changes in $[\text{Ca}]$. Fig. 1C and D show the time course of the change in I_{Na} peak amplitude during $[\text{Ca}]$ changes. The membrane potential during the test pulse was -30 mV. The Na permeability mechanism was almost fully turned on in 0.27 mM-Ca whereas it was only partially so in 14.8 mM-Ca. The change in amplitude of I_{Na} therefore indicated the change in the P_{Na} -potential relation caused by the change in $[\text{Ca}]$ of the bulk of the external solution. The half-time of this effect of $[\text{Ca}]$ changes was 200 – 300 ms or about twice as long as that of $[\text{Na}]$ changes. In a similar experiment on another fibre the half-time for the $[\text{Na}]$ effect was 100 ms when going from low $[\text{Na}]$ to high as well as when switched back to low $[\text{Na}]$. The half-time for the effect of $[\text{Ca}]$ changes was about 300 ms, i.e. about three times as long.

A high repetition rate of test pulses (20 pulses per s) was required to obtain a resolution of the time course of the I_{Na} changes. A slow decrease in peak P_{Na} appeared at this high repetition rate. This has recently been shown to be related to a slow inactivation process (Fox 1975; Rehm 1976). These changes introduced an uncertainty in the judgement of the effects of the peak P_{Na} amplitude corresponding to the half-time of the ionic concentration

change. The difference between the half time at the change from low to high [Ca] and the half time from high to low [Ca] in Fig. 1 C and D might consequently be apparent only.

A slower rate of change of the peak I_{Na} associated with the change in [Ca] compared to that associated with the change in [Na] was further observed when simultaneous changes [Na] and [Ca] were performed. Fig. 1 E shows a change to high concentrations and Fig. 1 F, the reverse change. The amplitude of the initial current depended on the sequence of solution change. The peak I_{Na} was larger soon after the change from low [Ca] and low [Na] to high [Ca] and high [Na] than in the steady state in any of these solutions. An overshoot of this magnitude was not seen at the solution change in the reverse sequence. It is quite possible that an application of a mixture of 2 solutions as used in these experiments is associated with I_{Na} of greater amplitude than is obtained in each of the basic solutions. In that case, however, transiently larger membrane currents should be recorded in both solution switches. By exclusion it is therefore concluded that the transient increase in the initial current shown in Fig. 1 E must depend on a slower effect of [Ca]-than of [Na]-exchange on the membrane. At the first solution change there was a transient increase in the peak I_{Na} when the high [Na] reached the membrane followed by the turn off of P_{Na} associated to the slower effect of high [Ca]. At the second solution change there was a rapid decrease in [Na] and the size of the inward current followed by a slower increase in the P_{Na} caused by the low [Ca].

In all fibres (about 20) tested in the way described by Fig. 1 E and F this sequence of events was observed. The potential of the test pulse, the [Ca]_o and the [Na]_o of the test solutions influenced the magnitude of the overshoot. When the solution changes were performed with repeated application of solutions with identical compositions no change occurred in the size of the initial current. The pulse duration was short in these experiments. A small continuous increase in the inactivation of the Na permeability mechanism could anyhow not be avoided. This effect developed in low [Ca]. The inactivation thus affected the amplitude of the initial current but in a direction opposite to that caused by the rapid solution changes.

Discussion

Potential clamp experiments were performed on single myelinated nerve fibres of *Xenopus laevis*. Short positive potential pulses were repeated with a frequency of 20 Hz while the amplitude of the associated membrane current was recorded. The size of the inward Na-current depends on the Na-permeability of the membrane and the electrochemical driving force for Na. Rapid changes in the external ionic composition were performed by rapid switches between solutions of different salt concentrations. The advantage of the described method for solution changes was that the dead space was minimal and that a distinct indication of the beginning of the solution change was obtained at the electric contact between the old and the new solution. The half time of the change in Na-current was estimated to be 100–150 ms at changes between solutions of different [Na]_o. On *Rana esculenta* Vierhaus and Ulbrich (1971) found with corresponding solution switches that the time constant of the exponential time course of the Na-current change was 30–50 ms, i.e. the half-time was 20–35 ms. The discrepancy between the two experimental measurements may be due to a species difference between fibres from *Rana* and *Xenopus* respectively.

An approximate estimate of the conditions for the solution change reveals that a volume much larger than the dead space (0.02 mm^3) flows past the fibre in about 20 ms. The new solution ought thus to have reached the fibre within this time. Excessive eddy currents might somewhat slow down the rate of solution change in the immediate vicinity of the fibre. In effect the solution switches were clearly slower than accountable to the solution pump. The ionic transport from an unstirred layer outside the fibre to the nodal membrane must likely be due to diffusion. Uncomplicated free diffusion in a plane sheath of $10 \mu\text{m}$ across would at a step concentration change at one surface change the concentration at the other surface (impermeable) to half complete in about 40 ms. The sheath thickness was somewhat arbitrarily taken as $10 \mu\text{m}$ ($6 \mu\text{m}$ nodal cement and $4 \mu\text{m}$ thickness of unstirred sea water). The effects measured were clearly slower. However the assumption of uncomplicated free diffusion might not hold for the structure outside the nodal membrane.

Investigations of the ultrastructure of the nodal region in frog fibres (Robertson 1959) and ventral foot fibres of the cat (Berthold 1968) show that the axonal membrane is covered by several structures in the nodal gap. These investigators further observed that the nodal gap was especially deep and narrow in large fibres as were used in the present study. This means that the free path for ions diffusing through the nodal gap can be very narrow at its narrowest part in the gap about 0.02 of the nodal area. This will obviously impede the ionic flux between the membrane and the bulk of the external solution. It further explains why the half-time for the effect of $[\text{Na}]$ changes is 100–150 ms instead of 40 ms as was theoretically calculated for the diffusion through the nodal gap.

The half-time of the effect of changes between solutions of different $[\text{Ca}]_o$ was 200–300 ms, which is twice that of the half-time of the effect of $[\text{Na}]$ changes. This difference is obviously not explained by the difference between the diffusion coefficients which is much less. It is, however, known that the nodal space, described by Hem and Young (1952) as the cementing disc, contains a substance, with the histochemical staining properties of a sulphated mucopolysaccharide (Langley and Landon 1967). These mucopolysaccharides have a large cation binding capacity (Langley 1969, 1970, Landon and Langley 1971). The combination of a limited diffusion and a large cation space in the nodal gap will limit the rate of change in cation concentration at the axonal membrane. This would also decrease the rate of $[\text{Ca}]$ changes more than the rate of $[\text{Na}]$ changes since the affinity for Ca is larger than for Na in polyions containing both sulphate and carboxyl residues (Scott 1967, Langley 1970). The concentration of negative groups in the nodal gap substance is unknown. In the mucopolysaccharide matrix of human cartilage it was estimated by Maroudas (1968) to 0.2 Eqv/l. A similar charge density in the nodal gap substance would by electrostatic binding of Ca considerably increase the effective Ca-space compared to that indicated by the volume of the nodal space. Except for mucopolysaccharides the nodal gap contains numerous villi- or finger-like processes extending perpendicular to the axon surface from the Schwann cells bordering the nodal gap. The membrane surface of these processes may also be negatively charged and thus contribute to an absorption of cations (polyvalent in particular) in the nodal gap. Due to a Ca-absorption in the nodal gap the local Ca content may be considerably higher than in the external solution. It should, however, be pointed out that Ca ions absorbed in this way are immobilized and should not effect the permeability properties of the axonal membrane.

change. The difference between the half-time at the change from low to high $[Ca]$ and half time from high to low $[Ca]$ in Fig. 1 C and D might consequently be apparent only.

A slower rate of change of the peak I_{Na} associated with the change in $[Ca]$ compared that associated with the change in $[Na]$ was further observed when simultaneous changes $[Na]$ and $[Ca]$ were performed. Fig. 1 E shows a change to high concentrations and Fig. 1 F the reverse change. The amplitude of the initial current depended on the sequence of solution change. The peak I_{Na} was larger soon after the change from low $[Ca]$ and low $[Na]$ to high $[Ca]$ and high $[Na]$ than in the steady state in any of these solutions. An overshoot of this magnitude was not seen at the solution change in the reverse sequence. It is quite possible that an application of a mixture of 2 solutions as used in these expts. is associated with I_{Na} of greater amplitude than is obtained in each of the basic solutions; that case, however, transiently larger membrane currents should be recorded in both solution switches. By exclusion it is therefore concluded that the transient increase in the initial current shown in Fig. 1 E must depend on a slower effect of $[Ca]$ -than of $[Na]$ -exchange on the membrane. At the first solution change there was a transient increase in the peak I_{Na} as the high $[Na]$ reached the membrane followed by the turn off of I_{Na} associated to the slow effect of high $[Ca]$. At the second solution change there was a rapid decrease in $[Na]$ as the size of the inward current followed by a slower increase in the P_{Na} caused by the low $[Na]$.

In all fibres (about 20) tested in the way described by Fig. 1 E and F this sequence of events was observed. The potential of the test pulse, the $[Ca]$ s and the $[Na]$ s of the solutions influenced the magnitude of the overshoot. When the solution changes were performed with repeated application of solutions with identical compositions no change occurred in the size of the initial current. The pulse duration was short in these expts. A continuous increase in the inactivation of the Na permeability mechanism could not be avoided. This effect developed in low $[Ca]$. The inactivation thus affected the amplitude of the initial current but in a direction opposite to that caused by the rapid solution changes.

Discussion

Potential clamp expts. were performed on single myelinated nerve fibres of *Xenopus laevis*. Short positive potential pulses were repeated with a frequency of 20 Hz while the amplitude of the associated membrane current was recorded. The size of the inward Na-current depends on the Na-permeability of the membrane and the electrochemical driving force for Na. Rapid changes in the external ionic composition were performed by rapid switches between solutions of different salt concentrations. The advantage of the described method for solution changes was that the dead space was minimal and that a distinct indication of the beginning of the solution change was obtained at the electric contact between the old and new solution. The half time of the change in Na-current was estimated to be 100-150 ms in changes between solutions of different $[Na]$ s. On *Rana esculenta* Vierhaus and Ulbricht (1971) found with corresponding solution switches that the time constant of the exponential time course of the Na-current change was 30-50 ms, i.e. the half time was 20-35 ms. The discrepancy between the two experimental measurements may be due to a species difference between fibres from *Rana* and *Xenopus* respectively.

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Investigations of the ultrastructure of the nodal region in frog fibres (Robertson 1959) and ventral root fibres of the cat (Berthold 1968) show that the axonal membrane is covered by several structures in the nodal gap. These investigators further observed that the nodal gap was especially deep and narrow in large fibres as were used in the present study. This means that the free path for ions diffusing through the nodal gap can be very narrow at a narrowest part in the gap about 0.02 of the nodal area. This will obviously impede the exchange between the membrane and the bulk of the external solution. It further explains why the half-time for the effect of $[\text{Na}]$ changes is 100–150 ms instead of 40 ms as was theoretically calculated for the diffusion through the nodal gap.

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During recent years several investigators have given evidence which supports the hypothesis that the mechanism for Ca-action is a neutralization of negative charges fixed on the surface of the axonal membrane. Such a neutralization could simply be due to the screening effect of a diffuse double layer of cations or in addition, to a specific Ca-binding. It is likely that the accumulation of Ca in the double layer would be rapid (see Grahame 1947) relative to the rates observed in the present investigation.

It is concluded that the slow action of Ca observed in the present investigation probably is related to the properties of the nodal gap. Especially important for the ionic exchange between the external solution and the axonal membrane is the cation binding property which has been ascribed to the nodal gap substance.

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The Effects of Variations in Extracellular Magnesium Concentration on Electrical and Mechanical Activity in Rat Portal Vein

By

STEFAN B SIGURDSSON and BENGT UVELIUS

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Abstract

SIGURDSSON S B and UVELIUS B. *The effects of variations in extracellular magnesium concentration on electrical and mechanical activity in rat portal vein.* Acta physiol scand 1977 99 368-376.

The effects of various concentrations of extracellular Mg^{++} on electrical and mechanical activity of the portal vein were studied. The integrated spontaneous contractile activity in the preparation was large in Mg^{++} -free solution, decreased to about 50% at 1.2 mM Mg^{++} and was almost completely abolished at 10 mM Mg^{++} . Each spontaneous contraction became smaller whereas contraction frequency was less affected. Second gap recordings showed that the reduced spontaneous mechanical output was associated with decreased electrical activity on increasing $[Mg^{++}]_o$. Increasing $[K^+]_o$ from 6 to 12 mM normalized the spontaneous mechanical activity in 10 mM Mg^{++} solution. Local registration of electrical activity with 3 extracellular glass capillary electrodes showed that inactive areas developed at the high Mg^{++} concentrations. These findings indicate that Mg^{++} exerts a hyperpolarizing action on the smooth muscle cell membrane and, at the highest concentration, interferes with intercellular propagation. $[Mg^{++}]_o$ in the range of 0-10 mM had no effect on amplitude of K^+ (12 mM) contractions at $[Ca^{++}]_o > 0.5$ mM. At $[Ca^{++}]_o < 0.5$ mM the amplitude decreased with increasing $[Mg^{++}]_o$. The latter observation indicates that Mg^{++} can interfere with the Ca^{++} -permeability of the depolarized cell membrane. 1 mM EDTA, 3 mM Na citrate, 0 mM Mg^{++} and 0 mM Ca^{++} gave lower intracellular Mg^{++} below the minimum concentration needed for contractile activity.

The extracellular magnesium concentration which may vary considerably under pathological conditions (see e.g. Walser 1967) affects blood flow resistance through a direct effect on vascular smooth muscle (Viveros and Somjen 1968). It was suggested by Altura and Altura (1974) that Mg -ions can affect the excitation-contraction coupling in vascular smooth muscle by participation in the regulation of the cell membrane permeability and buffering capacity of calcium. It has been demonstrated that isolated actomyosin from smooth muscle is dependent on Mg ions for ATPase activity (Murphy *et al.* 1969). Magnesium plays a fundamental role in many biochemical reactions including the release and transfer of phosphate groups (see e.g. Livingstone and Wacker 1971).

The aim of the present study was to obtain further information on the effect of variations

intracellular magnesium concentrations on smooth muscle by recording the electrical and mechanical activity in the isolated rat portal vein. An attempt was also made to lower intracellular Mg^{++} by chelating the ion with EDTA.

A preliminary report of the present study has been published earlier (Sigurdsson and Holm 1975).

Material and Methods

Just 48 portal vein preparations from rats of the Sprague-Dawley strain, weighing 200 to 300 g were used. The animal was killed by blow on the neck and the vein was dissected out. The preparations used in experiments where only isometric tension was recorded were about 0.5 cm long. They were mounted in 1 ml mineral oil between a muscle holder and force transducer (Grass FTO3). The muscles were stretched to and maintained at a preload of about $4 \cdot 10^{-6}$ N and allowed to accommodate for at least 12 h before the beginning of the experiments. For simultaneous recording of electrical and mechanical activity about 2 cm of the portal-muscular vein was removed and mounted in a sucrose gap apparatus as described by Ashworth *et al.* (1967). The electrical signals were amplified and recorded in an AC mode and the number of spikes counted electronically as described by Johansson and Mellander (1975).

For simultaneous recording of electrical activity from three different sites on the muscle, about 1 cm of the vein was dissected out and cut open longitudinally. It was stretched flat between a muscle holder and force transducer (Grass FTO3) at a preload level of $4 \cdot 10^{-6}$ N. Three glass capillary electrodes containing physiological salt solutions were then placed on different sites of the preparation (for further details see Lund and Stepp 1970). The AC coupled signals were amplified and recorded on the Grass polygraph.

The bathing media in the experiments were based on Tris buffered solutions of the following composition in mM: NaCl 120, KCl 6, glucose 11.5 and tris (hydroxymethyl) aminomethane (Trizma base, Segna Chemical Co.). The solution was titrated with HCl to pH of 7.4 at 37°C, and it will in the following be referred to as "Na-tris". $CaCl_2$ and $MgCl_2$ are then added to this solution to obtain desired concentrations. The accommodation solution was Na-tris with 2.5 mM Ca^{++} and 1.2 mM Mg^{++} . A K-tris solution all NaCl replaced its cationic elements of KCl. 1 mM EDTA was used to get a solution low (10^{-4} M) in Ca^{++} (Rutledge *et al.* 1968). All solutions were bubbled with 100% O_2 .

In order to avoid the possible influence of noradrenaline release from the adrenergic nerve supply by lower ion changes, phentolamine (Dibenzylamine, Boehr, Kline & Freck, generously supplied by Boehr & Co, Mainz, Sweden) was added to the accommodation solution to a concentration of 10^{-6} M or less so response to added noradrenaline was obtained.

Results

The effect of Mg^{++} on the spontaneous mechanical activity of the rat portal vein was studied. The spontaneous phasic contractions in Na-tris with 2.5 mM Ca^{++} and 0 Mg^{++} are seen in Fig. 1 A. Fig. 1 B-E shows the results of increasing the Mg^{++} -concentration, in steps from 0.2 mM (B) to 1.0 mM (E). The muscle had been in the respective solution for 1/2 h when the recordings were obtained. It is clearly seen that the amplitude of the phasic contractions is diminished considerably by increasing Mg^{++} whereas the contraction frequency is almost unchanged. This effect was not due to changes in osmolality as osmotic compensation by adding sucrose to the 0 Mg^{++} solution or reducing NaCl in the Mg^{++} -high solutions did not affect the response.

Fig. 2 shows simultaneous recordings of mechanical and electrical activity in the sucrose gap apparatus. The recordings are from top to bottom: isometric active force, integrated active force sampled over 1 min periods, number of spikes, and AC-recording of electrical activity. Panels 2 A-D show the results of increasing the Mg^{++} -concentration from 0.2 to 1.0 mM. The contraction amplitude diminishes as in Fig. 1 and, concomitant with this, a

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The aim of the present study was to obtain further information on the effect of variations

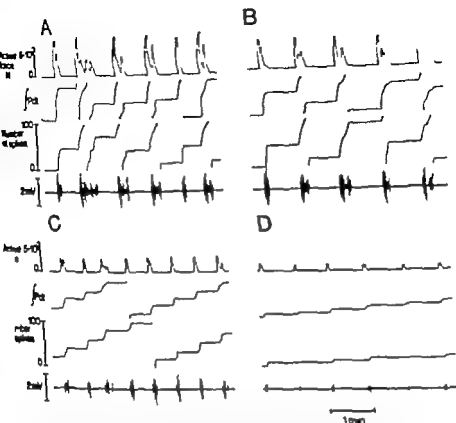


Fig. 2. Simultaneous recording of mechanical and electrical activity. The tracings from top to bottom: resting active force, integrated active force, number of electrical spikes and AC recording of electrical activity. The figure shows the inhibitory effect on electrical and mechanical activity of stepwise increases in $[Mg^{2+}]_0$ from 1.2 (A) to 2.4 (B), 4.8 (C) and 10 mM (D).

under conditions where no spikes can appear the effect of raising $[Mg^{2+}]_0$ on the amplitude of K⁺-contractures was studied. Fig. 1 shows that the sustained contracture amplitude was reduced when $[Mg^{2+}]_0$ was increased from 0 to 10 mM in K⁺-trials with 2.5 mM Ca²⁺. The fast, phasic part of the K⁺-response decreased like the spontaneous activity as the $[Mg^{2+}]_0$ was increased. A quantitation of the Mg^{2+} effect on the K⁺-contractures in different $[Ca^{2+}]_0$ is shown by the broken lines in Fig. 4. The amplitude of the sustained K⁺-contractures is not altered by increase in $[Mg^{2+}]_0$ for extracellular Ca^{2+} -concentrations between 0.5 and 10 mM, whereas for $[Ca^{2+}]_0$ 0.5 mM decrease in response is seen for the higher $[Mg^{2+}]_0$. The smaller the $[Ca^{2+}]_0$, the more sensitive does the muscle become to changes in $[Mg^{2+}]_0$.

As the solution with 0 mM Mg^{2+} was only nominally Mg^{2+} -free it was of interest to decrease $[Mg^{2+}]_0$ further. This was done by adding 1 mM EDTA to the nominally Mg^{2+} - Ca^{2+} -free Na-tris. EDTA forms complexes with divalent cations. According to Hubbard *et al.* (1968) the concentration of free Mg^{2+} and Ca^{2+} in this solution is below 10^{-8} and 10^{-9} M respectively. When placed in this nominally Mg^{2+} - Ca^{2+} -free solution with 1 mM EDTA the muscle

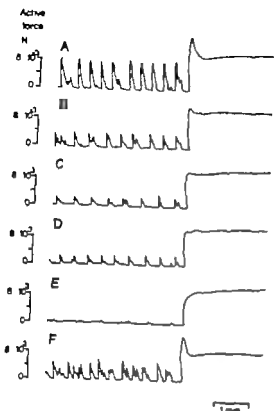


Fig. 1 In the left half is shown the effect on the spontaneous activity of one preparation by increasing the $[Mg^{2+}]_i$ stepwise from 0 (A) to 1.2 (B), 3.4 (C), 4.8 (D) and 10 mM (E). I (F) is shown how the muscle recovers in solution with 0 mM Mg^{2+} . In the right half are shown potassium contractions in the respective solutions.

corresponding decrease in the number of spikes is demonstrated when $[Mg^{2+}]_i$ is raised. To see if this membrane effect was due to hyperpolarization the concentration of K⁺ in Na-tris 2.5 mM Ca^{2+} 10 mM Mg^{2+} was increased from 8 to 12 mM. The spontaneous electrical and mechanical activity then became normalized, i.e. as in Na-tris 2.5 mM Ca^{2+} 1.2 mM Mg^{2+} solution (not shown in the figure).

The electrical activity seen in Fig. 2 shows the mass activity in the whole muscle. From this kind of registration it is not possible to determine whether all parts of the preparation participate in the phasic contractions. Therefore one cannot exclude the possibility that besides the diminished number of spikes per burst, there may be blocking of propagation when $[Mg^{2+}]_i$ is raised. By using three extracellular glass capillary electrodes placed on different parts of the preparation, it was possible to obtain information on how the electrical activity propagated along the muscle. Fig. 3 A shows that the areas under the three electrodes were all activated and showed similar burst durations during a contraction when $[Mg^{2+}]_i$ was 1.2 mM. When $[Mg^{2+}]_i$ was increased the propagation was impaired. First in solution with 5 mM Mg^{2+} (B) the number of spikes in each burst decreased as it propagated along the preparation and finally in solution with 10 mM Mg^{2+} some of the bursts failed to propagate to the electrode most remote from the pacemaker area (C).

A quantitation of the Mg^{2+} effect on the spontaneous activity in different $[Ca^{2+}]_i$ is shown by the full lines in Fig. 4. The integrated spontaneous mechanical activity decreased rapidly when $[Mg^{2+}]_i$ was increased for all $[Ca^{2+}]_i$ studied.

In order to see if Mg^{2+} would influence the degree of activation of the contractile apparatus

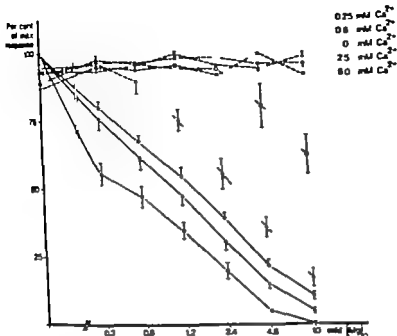


Fig. 4 Quantitative effects of stepwise increases in $[Mg^{2+}]_i$ from 0–10 mM in solutions with various $[Ca^{2+}]_i$ expressed as percentages of the maximal force. Broken lines show effects on potassium contractures, full lines effects on integrated spontaneous activity. Each point represents the mean of five or more measurements. Vertical bars represent S.E.

1.2 mM Mg^{2+} and became spontaneous active again. The other (lower recording) was placed in Na-tris 2.5 mM Ca^{2+} but 0 mM Mg^{2+} as before and nothing happened (E). In F the muscles were depolarized in K-tris 2.5 mM Ca^{2+} 1.2 mM Mg^{2+} (upper recording) and K-tris 2.5 mM Ca^{2+} 0 mM Mg^{2+} (lower recording) after a 5 min period in Na-tris 0 mM Ca^{2+} 1.2 mM Mg^{2+} (upper recording) and Na-tris 0 mM Ca^{2+} 0 mM Mg^{2+} (lower recording). The muscle which had had Mg^{2+} (upper recording) developed a relatively strong contracture, but the Mg^{2+} -depleted muscle (lower recording) gave only a very small response. In G is shown how both muscles recovered in Na-tris 2.5 mM Ca^{2+} 1.2 mM Mg^{2+} .

Discussion

In 1968 Verroen and Somjen found that resistance vessels in the gracilis muscle of the dog in vivo dilate when exposed to a high concentration of Mg^{2+} in the blood. They thought that this dilatation was caused by a direct action of free Mg^{2+} on the smooth muscle cells of the vessels by preventing the entry of Ca^{2+} from the extracellular fluid into the muscle cell or in some other way interfering with excitation-contraction coupling. These results were confirmed in vitro by Altura and Altura (1974) and Jurevics and Carrier (1973). Our results show that increased $[Mg^{2+}]_i$ has an inhibitory effect on the spontaneous electrical and mechanical activity of the rat portal vein. Maximal integrated force was obtained in Mg^{2+}

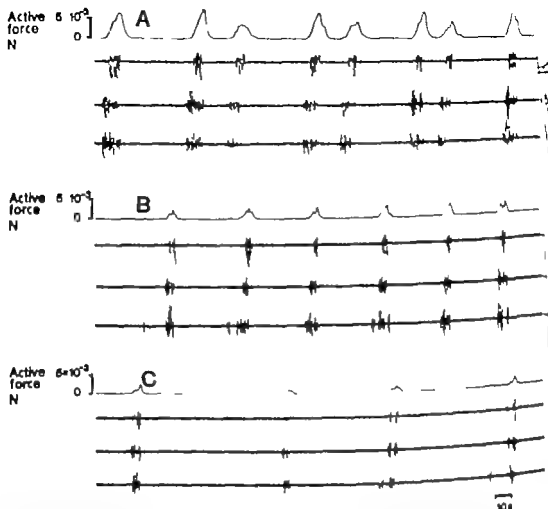


Fig. 3 Recording of mechanical activity and electrical activity from 3 electrodes placed on different sites on the muscle. The muscle was in solutions with 1.2 mM Mg^{2+} (A), 4.8 mM (B), and 10 mM (C). With increasing $[Mg^{2+}]$, there is a decrease in spike activity and in C impaired spike propagation is seen.

lost spontaneous activity as would be expected merely from the low $[Ca^{2+}]_o$. After 1 h in this medium, the muscles were placed in Na-tris with 2.5 mM Ca^{2+} but with no Mg^{2+} for 1/2 h. No activity appeared. When Mg^{2+} was added the muscle again became spontaneously active. The result of one such experiment is shown in Fig. 5. Recordings were obtained from 2 preparations (upper and lower tracings). In A the muscles were spontaneously active in Na-tris solution with 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+} . The muscles were then placed in Na-tris 0 mM Ca^{2+} 1.2 mM Mg^{2+} for 5 min and then in K-tris with 2.5 mM Ca^{2+} 1.2 mM Mg^{2+} (arrow). Contractions developed (B). After this the muscles relaxed in Na-tris 0 mM Ca^{2+} 1.2 mM Mg^{2+} for a few min and they were then transferred to nominally Ca^{2+} Mg^{2+} free Na-tris with 1 mM EDTA (C). One hour later the solution was changed to Na-tris 2.5 mM Ca^{2+} 0 mM Mg^{2+} . No spontaneous activity appeared and only a very small contraction was seen when the muscles were depolarized (arrow) in K-tris 2.5 mM Ca^{2+} 0 mM Mg^{2+} (D). One of the muscles (upper recording) was now placed in Na-tris 2.5 mM Ca^{2+}

lects the excitation-contraction coupling (Altura and Altura 1974, Jurivics and 1973)

by *et al.* (1969) compared the Mg^{2+} dependence of skeletal and arterial smooth myogenesis. They found that for skeletal muscle the response of the contractile reached maximum at about 10^{-4} M Mg^{2+} when the Ca^{2+} and ATP concentrations optimal. They did not succeed in defining precisely the Mg^{2+} dependence for arterial myogenesis but found the highest rates of ATP-use activity when the free- Mg concentration reached 10^{-4} M.

ity (1971, 1974) has found on the rat tail artery that only half of the tissue Mg is lost the smooth muscle is incubated in Mg -free Krebs for 24 h. In this Mg -free solution was first a rapid loss of tissue Mg depending on the original $[Mg^{2+}]_i$, followed by a slow loss related to the content of intracellular compounds forming complexes with Mg^{2+} . Mg^{2+} is lost in Mg - Ca -free solution because external Ca^{2+} markedly decreases the permeability of the cell membrane to passive movements of Mg^{2+} . Palaty also found in electrically active muscle that the $[Mg^{2+}]_i$ is maintained at a level of about 10^{-4} M. This is much lower than in his normal Krebs solution indicating that the muscle appears to maintain an active Mg^{2+} extrusion mechanism.

In our experiments the portal vein could maintain its increased contractile activity in the solution for hours. When also Ca^{2+} was removed from this solution and 1 mM EDTA was added, the muscle relaxed. 1 mM EDTA in nominally Mg - Ca -free solution sets the free $[Mg^{2+}]$ below 10^{-8} M and free $[Ca^{2+}]$ below 10^{-8} M (Hubbard *et al.* 1968). The muscle after 1 h in this solution was placed in the Mg -free Na-tris with 2.5 mM Ca^{2+} remained inactive. Contractile activity reappeared when 1.2 mM Mg^{2+} was added. It seems that during the period in Mg - Ca -free solution with 1 mM EDTA cell Mg is lowered below the Mg^{2+} concentration which is needed for spontaneous activity. It is shown in Table 3 that a depolarization of the muscle by K-tris 2.5 Ca^{2+} 0 Mg^{2+} gave only a contraction about 10% of that obtained when 1.2 mM Mg^{2+} had also been added to the solution.

This present study was supported by grants from the Swedish Medical Research Council (28-14 X), from the Medical Faculty, University of Lund, and from AB H&S, Göteborg, Sweden. Thanks are due to Mrs Monica Lundahl, Mrs Monica Hurdénholm and Miss Ina Gustafsson for able technical assistance.

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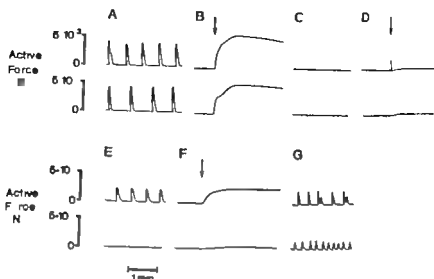


Fig. 5 The effect of a Mg-Ca-free solution with 1 mM EDTA on the spontaneous activity and potent contractions in two preparations of the rat portal vein. For details see text.

free solution, and a stepwise increase in $[Mg^{2+}]_o$ decreased the mechanical and electrical activity (Fig. 1). There was a decrease in contraction amplitude and in some preparations a tendency to a decrease in the frequency of contractions. At 10 mM $[Mg^{2+}]_o$ the preparation usually had no spontaneous activity at all.

In the sucrose-gap experiments it was clearly shown that increasing $[Mg^{2+}]_o$ within the low concentration range led to comparable inhibition of electrical and mechanical activity. At the highest Mg concentration (10 mM) the spikes had become so small that the spike counter underestimated their number. The decrease in mechanical activity is most likely due to inhibition of the electrical activity. A possible membrane effect of Mg could be an impairment of conductance between the cells. Our results with 3 glass capillary electrodes showed that for the higher Mg concentrations the spike propagation was impaired (Fig. 3 C). The results described above can be explained largely as an hyperpolarizing effect. Mg^{2+} as a moderate increase in $[K^{+}]_o$ (12 mM) eliminated the inhibitory action of Mg^{2+} .

It was considered possible that Mg besides the hyperpolarizing action on the spontaneously active muscle might influence the degree of activation of the potassium depolarized muscle. It was found (Fig. 1 and 4) that Mg in concentrations between 0 and 10 mM did not influence the steady amplitude of the potassium induced contraction when $[Ca^{2+}]_o$ was between 0.5 and 5 mM. Only when $[Ca^{2+}]_o$ was 0.5 mM did increases in $[Mg^{2+}]_o$ affect the contraction amplitude. It is probable that Mg^{2+} can affect Ca^{2+} permeability for $[Ca^{2+}]_o < 0.5$ mM. For higher $[Ca^{2+}]_o$ intracellular Ca^{2+} concentration might become larger than that needed for complete activation of the contractile apparatus. A moderate decrease of Ca^{2+} permeability would therefore not have any effect on the contractile output.

It was noted that the initial fast phase of the K response which is thought to be the result of liberation of superficially bound Ca^{2+} (Sigurdsson, Uvelius and Johansson 1975) became less prominent when the $[Mg^{2+}]_o$ was increased. It is likely that Mg affects the spike appearing during the fast phase (Sigurdsson, Uvelius and Johansson 1975) or in some other

ffects the excitation-contraction coupling (Altura and Altura 1974 Jurevics and Wier 1973)

Marple *et al.* (1969) compared the Mg^{2+} dependence of skeletal and arterial smooth muscle actomyosin. They found that for skeletal muscle the response of the contractile state reached maximum at about 10^{-4} M Mg^{2+} when the Ca^{2+} and ATP concentrations were optimal. They did not succeed in defining precisely the Mg^{2+} dependence for arterial myosin but found the highest rates of ATPase activity when the free-Mg concentration approximated 10^{-4} M.

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Quantitative Evaluation of the Blood-Brain Barrier Capacity to Form Dopamine from Circulating L-DOPA

By

I. E. HARDEBO, L. EDVINGSSON, CH. OWMAN and E. ROSENGREN

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Abstract

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The ability of the blood-brain barrier to form dopamine from increasing doses of systemically administered L-DOPA has been studied *in vivo* by combination of chemical decarboxylation of dopamine, and histochemical and cytofluorometric measurements of L-DOPA and dopamine. The break-through of DOPA into the circulation (into the brain pericythemia) via the carotid blood-brain barrier was estimated by comparing the amount of newly formed dopamine in the caudate nucleus-putamen and in the cerebellum. The capillaries were found to efficiently trap L-DOPA in their walls, and an upper limit was reached (at an administered dose of 100 mg/kg of L-DOPA). It could be estimated that approximately 3% of the total dose of L-DOPA given was decarboxylated by the blood-brain barrier. The possible influence by the regional differences in perfusion of the two regions seen after administration of L-DOPA was ruled out in measurements of local cerebral blood flow using the ^{14}C -ethanol technique.

The capillaries in the central nervous system are capable of decarboxylating certain amine precursors, such as dihydroxyphenylalanine (DOPA) (Bertler *et al.* 1966, Owman and Rosengren 1967, Barthollet *et al.* 1971), 5-hydroxytryptophan (Bertler, Falck and Rosengren 1964), and dihydroxyphenylserine (Constantinidis *et al.* 1975), thus impeding the passage of these amino acids into the brain. This blood-brain barrier property appears to be a general feature among several laboratory animals studied, as well as in humans (Hardebo *et al.* 1976). In peripheral tissues these amine precursors pass freely through the vessel walls into the surrounding tissues, probably because the vessel wall is devoid of a decarboxylating capacity (Owman and Rosengren 1967). The enzymatic barrier mechanism is interesting both from a general biological standpoint, constituting one special type of blood-brain barrier function (see Rapoport 1976), and clinically in the medical therapy of Parkinson disease (Cotzias, Papavasiliou and Gellera 1969).

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Fig. 1. Animal pretreated with 5 mg/kg of reserpine and 100 mg/kg of nialamide. 20 min after the injection (60 mg/kg) of L-DOPA, an intense green fluorescence develops in the brain capillary walls. Note the striking array of the capillary network in the caudate nucleus (a) and the cerebellum (b). The parenchymal "background" is both regions essentially non-fluorescent. Magnification, 90.

Results

Fluorescence microscopy of the caudate nucleus from non-treated control animals showed a intense, green fluorescence in the parenchyma deriving from the very dense network of these dopamine-containing axon terminals (cf. Fuxe 1965; Lindvall and Björklund 1974). The parenchyma in the cerebellum, on the other hand, was essentially non-fluorescent and only a small number of isolated green-fluorescent noradrenaline axons was present in the plexiform layer. The walls of the parenchymal blood vessels in both regions were non-fluorescent, except for the presence of the perivascular sympathetic nerve fibres (cf. Owman *et al.* 1974).

Reserpine treatment was used with the aim of depleting the dopamine from the caudate nucleus to make it better comparable with the cerebellum—which contains no measurable dopamine (Berder *et al.* 1966)—in terms of the basal catecholamine level. A dose of 5 mg/kg (as shown from a series of pilot expts.) reduced the concentration of dopamine after 4 h to a mean of 0.45 ± 0.15 $\mu\text{g/g}$, which is approximately 5% of the normal dopamine level.

Administration of 60 mg/kg of L-DOPA to reserpinized animals, in which monoamine oxidase had been inhibited by nialamide, resulted in a green fluorescence of equally high intensity in the capillary walls of the two regions analyzed, whereas the parenchyma remained essentially non-fluorescent (Fig. 1). It has previously been shown that the capillary fluorescence reflects the local formation of dopamine from the exogenous precursor amino acid (Berder *et al.* 1966).

The extent of dopamine formation in the cerebellar and striatal tissues was followed after administration of increasing doses of L-DOPA (Fig. 2). It can be seen that a similar amount was formed in both regions up to a dose level of 100 mg/kg of L-DOPA, after which the concentration in the cerebellum levelled out, despite the administration of increasing doses of precursor. In the caudate nucleus-putamen the increment in the dopamine formation continued, so that a markedly and significantly higher concentration was achieved upon 140 to 160 mg/kg of L-DOPA as compared with cerebellum (Fig. 2).

It can be assumed that the decarboxylation capacity in the brain capillary wall has a limit which means that the amino acid, for example L-DOPA, penetrates into the brain parenchyma at particularly high circulating concentrations. In regions, such as cerebellum, containing no dopamine-storing neurons (Lindvall and Björklund 1974), where the formation of measurable amounts of dopamine thus can be expected to take place only in the capillary walls, the concentration of dopamine will come to a maximum when the limit is reached. However, in other areas, such as the caudate nucleus, the dopamine concentration will continue to increase beyond this limit, namely when the large amount of dopamine in the nerves in this region (Fuxe 1965; Lindvall and Björklund 1974) becomes exposed to L-DOPA passing into the brain parenchyma. This hypothesis was tested in the present study by administration of increasing doses of L-DOPA to rats, followed by chemical determinations of the amount of newly formed dopamine in the caudate nucleus and cerebellum.

Material and Methods

Animals. The study was performed on female Sprague-Dawley rats weighing 190–200 g and fed with standard pellets and tap water *ad lib*. One group of 10 non-treated animals served as controls. The remaining 100 animals were pretreated with 5 mg/kg i.p. reserpine (Serpasil, Ciba) followed 4 h later by the monoamine oxidase inhibitor nialamide (Niamid, Pfizer 100 mg/kg i.p.). One hour later 88 animals were injected with increasing doses (70, 40, 60, 80, 100, 140, 200 and 300 mg/kg i.p.) of L-DOPA (L-3,4-dihydroxyphenylalanine, Sigma) fully dissolved in 0.9% saline, and killed 20 min later.

Fluorescence microscope. Immediately after decapitation, small tissue pieces were dissected out from cerebellar hemisphere and caudate nucleus-putamen on the right side, frozen to the temperature of liquid nitrogen and further processed for fluorescence monoamine histochemistry according to Björklund and Ojman (1972). The paraformaldehyde used in the histochemical procedure has previously been calibrated with it of 70% humidity. The formaldehyde-induced, histochemically visible fluorescence in both catecholamines and DOPA have the same spectral characteristics, with excitation/emission maxima at 410/470–480 nm (Björklund *et al.* 1972), and exhibit a green light under the optical conditions used.

The cerebrovascular system was outlined in some of the animals by perfusion with India ink (Eklöf 1966) through the left ventricle of the heart under ether anaesthesia.

Cytofluorometry. Specimens processed for fluorescence histochemistry as above were used. The cerebellar and striatal tissues were embedded side-by-side in the same paraffin block and sectioned simultaneously at 5 µm thickness. The intensity of the formaldehyde-induced fluorescence in the capillary wall and in adjacent parenchyma was compared, using a Leitz microspectrograph (Björklund, Chingir and Falck 1971) at 250× objective magnification. A mercury lamp (Osram HBO 200 W/2) was used as light source, monochromator at the excitation side was fixed at 405 nm, and a filter with its edge at 430 nm was used as barrier. The entrance slit of the monochromator at the emission side was open between 460 and 480 nm. The photomultiplier signals expressing relative fluorescence were read on a galvanometer. The procedure of locating the fluorescent structure, focusing, and obtaining the measurement response in less than 30 s, during which time the UV photodecomposition by the light source has been shown to amount only about 5% of the initial fluorescence intensity (Jonsson 1971).

Chemical determinations. After killing of the animals, the entire cerebellum and the caudate nucleus-putamen tissue of both sides were dissected out and homogenized separately in ice-cold 0.4 N perchloric acid for fluorometric determination of dopamine according to Aston and Sayre (1964). One rat was used for each determination.

Blood flow measurements. Cerebral blood flow was measured by radioactive clearance technique according to the procedure described by Eklöf *et al.* (1974), using ethanol- C^{14} as indicator. The scintillation countings were carried out on cerebellar preparation weighing approximately 100 mg and consisting of both grey and white matter and striatal preparation (about 25 mg) consisting of caudate nucleus-putamen tissue from both sides.

Statistics. Mean values were compared according to Student's *t*-test. A Hewlett Packard desk computer was used in the calculations.

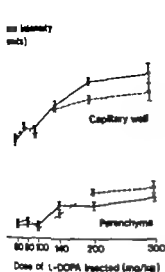


Fig. 3

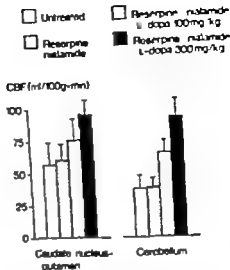


Fig. 4

Fig. 3. Crystalline measurement of formaldehyde-induced fluorescence (of DOPA and dopamine) in capillary wall and parenchyma from caudate nucleus-putamen (●—●) and cerebellum (○—○) of rats using the same technique as described in Fig. 2. Mean \pm SEM of 8 measurements at each dose level.

Fig. 4. Regional cerebral blood flow (CBF) in caudate nucleus-putamen and cerebellum of untreated animals and after administration of two dose-levels of L-DOPA to animals pretreated with reserpine and amphetamine. Mean \pm SEM, 5 determinations in each group.

as an equally low endogenous base level of the amine in the two structures. Therefore, reserpine was given in a series of pilot experiments to find out the lowest dose that, during the longest time of treatment, would cause sufficient reduction in the high dopamine content of the striatal tissue. Although a dose of 5 mg/kg after 20 h resulted in a complete depletion of striatal dopamine, a shorter time of 4 h (reducing the dopamine level by approximately 50%) was chosen because the animals were considered to be in too bad general condition for the long-term reserpization. Subsequent treatment with a monoamine oxidase inhibitor (naloxone) was given to avoid break-down of the amine formed from the exogenous L-DOPA, in the brain parenchyma as well as in the capillary walls (Bertler *et al.* 1966). In view of the working hypothesis mentioned in the introduction, the results would indicate that the upper limit of entrapment capacity for L-DOPA in brain capillaries is reached (at an intraperitoneal dose level of 100 mg/kg given 20 min before the animal was killed). At higher doses, part of the amine precursor appears to leak beyond the capillary wall into the brain parenchyma: the increment in the concentration of dopamine in the caudate nucleus-putamen continues (Fig. 2), reflecting that dopamine is now being formed not only in the capillary wall but also in the parenchyma of this tissue. In the cerebellum there was no corresponding increase after the upper limit of entrapment capacity had been reached, the tendency to a slight increment in dopamine formation here at the highest L-DOPA dose may be due to the appearance of measurable amounts of dopamine in the (non-dopaminergic)

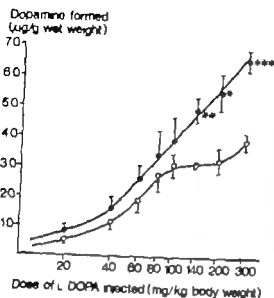


Fig. 2. Semilogarithmic representation of dopamine formed (expressed as μg per gram wet weight) caudate nucleus-putamen (●-●) and cerebellum (○-○) of rats pretreated with reserpine 4 hrs before L-DOPA in increasing doses, and killed 20 min later. Mean \pm SEM, 6 fluorimetric determinations at each dose level. Dopamine animals receiving no L-DOPA, caudate nucleus-putamen, $0.45 \pm 0.15 \mu\text{g/g}$; cerebellum, non-measurable level. Comparison of matching means in the two flanges by Student's *t*-test: * $p < 0.001$; $p < 0.01$ other differences non-significant.

In the fluorescence microscope a capillary fluorescence could be detected already at lowest L-DOPA dose (20 mg/kg) though the intensity of the emitted light was only weak. It became higher with increasing doses of exogenous precursor until 100–140 mg/kg at which no further increment could be seen. The "background" fluorescence in the parenchyma was low up to the same dose level of L-DOPA after which a clear increase, similar in the two regions, could be detected at the two highest doses of L-DOPA.

The density of the capillary network was compared in the cerebellar and striatal tissues on the basis of the arrangement of fluorescent capillary walls as well as the appearance of ink loaded microvessels. No difference was found in either white or grey matter in the two regions (Fig. 1).

In order to obtain a more quantitative estimation of the histofluorescence changes in the capillary wall and parenchyma, intensity measurements were carried out on sections from the two regions. It can be seen from Fig. 3 that the capillary wall fluorescence was significantly higher than the corresponding parenchymal fluorescence. Further in the cerebellar and striatal tissues the pattern of increase in fluorescence with increasing doses of L-DOPA was similar in capillaries and parenchyma, respectively.

The blood flow in the caudate nucleus-putamen and cerebellum was measured in untreated controls and after the drug injections. The flow under normal conditions (Fig. 4) was lower in the cerebellum, though the difference was not statistically significant. Combined treatment with reserpine followed by nialamide had no effect on flow in either region. However when L-DOPA was added to the treatment an increased blood flow could be measured; this increment was more pronounced in the cerebellar tissue.

Discussion

In order to allow for comparison of the dopamine formation in the preparation of caudate nucleus-putamen and the cerebellar tissue after the L-DOPA injections, it was desirable to

L-DOPA used in the present study. It could be expected that a plateau in dopamine accumulation should form not only in the cerebellum but also in the striatal tissue.

Attempts have previously been made to determine the capacity of the enzymatic barrier as well as the basis of semiquantitative estimation of fluorescence intensities using the formaldehyde method (Berter *et al.* 1966, Dawson and Laszlo 1971, de la Torre and Mullan 1971). Varying results have been reported, apparently because the approaches used have been less precise than the combination of techniques applied in the present experimental model.

The efficiency of the blood-brain barrier to impede the passage of L-DOPA into the brain as a decarboxylating mechanism, in addition to the decarboxylation activity in the peripheral tissues, have required high doses of L-DOPA in the medical treatment of Parkinson's disease with disturbing side-effects as a frequent result. Partly based on the knowledge about the mechanism of this special type of enzymatic barrier (see Owman *et al.* 1974, Hardebo *et al.* 1976) it has now been possible to overcome, to a considerable extent, these problems: a laboratory-made inhibitor (Ro 4-4602 or MK 486), effective in peripheral tissues as well as in the capillary cells but only to a negligible extent in the brain parenchyma, is added to L-DOPA in the therapy. Under these conditions, the amine precursor can be administered in lower doses, reducing the incidence of undesired side-effects (Mars 1973, Mifler and Wiener 1974).

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monoamine nerves exposed to the L-DOPA leaking into the cerebellar parenchyma. On the basis of the maximum amount of dopamine formed in the cerebellum (3.12 µg/g) occurring at an i.p. dose of 100 mg/kg of L-DOPA it can be estimated that approximately 3% of the exogenous amino acid is decarboxylated in the blood-brain barrier (whereas the remainder of the conversion takes place in peripheral tissues).

There is evidence already at the level of fluorescence microscopy both in the present and previous (Bertler *et al.* 1966) studies, that the fluorophores which can be visualized after administration of L-DOPA at low doses are located only in the microvessel walls, whereas at high doses of L-DOPA an increasing intensity of fluorescence becomes visible also in the parenchyma, where it has a diffuse localization. A similar diffuse parenchymal fluorescence starts to appear also at low L-DOPA doses following inhibition of the decarboxylase (in the capillary walls) (Bertler *et al.* 1966). In spite of these fluorescence microscopic findings, it would still be possible that at least part of the steady increase in striatal dopamine formation measured chemically (Fig. 2) would be explainable by a higher decarboxylase activity in the capillary walls of the striatum compared with the cerebellar vascular beds. However, this is contradicted by the cytofluorometric measurements showing that the pattern of increasing fluorescence intensity was very similar in the capillary walls from the two regions (Fig. 1). Also the studies by Wade and Katzman (1975) support the view that the decarboxylase activity at the level of the capillary wall is similar in various brain regions (having equal brain uptake indices for L-DOPA after a single capillary circulation). The intensity of capillary fluorescence continued to increase even when the limit of capillary decarboxylation capacity had been reached. This can be explained by the continued capillary uptake of L-DOPA whose fluorescence is added to the dopamine fluorescence in the capillary endothelium. The cytofluorometric measurements of the intensity of the diffuse fluorescence in the parenchyma agreed with the fluorescence microscopic observations in that a definite increase in this fluorescence did not occur until a dose of above 100 mg/kg of L-DOPA (i.e. when the limit of capillary decarboxylation capacity had been reached). The difference between the curves for the fluorometric determinations (Fig. 2) and the cytofluorometric measurements (Fig. 3) thus lies in the fact that the former shows dopamine specifically formed simultaneously in the microvasculature walls and the parenchyma whereas the latter only measures the fluorescence selectively in the microvasculature walls and parenchyma, and cannot discriminate between the fluorophores of DOPA and dopamine (Björklund *et al.* 1972).

The fluorescence microscopic analysis indicated that the density of the capillary plexus was equal in the two regions studied. The regional measurements of cerebral blood flow moreover revealed that the increase in flow upon L-DOPA injection was more pronounced in the cerebellum. This shows that the significantly higher dopamine formation in the striatum at L-DOPA doses above 100 mg/kg could not simply be due to a better perfusion of this region compared with the cerebellum.

In isolated perfused rat brains, Horst *et al.* (1974) have found that the dopamine accumulation in the tissue during perfusion with increasing concentrations of L-DOPA does not proceed in a continuous elevation, but progresses through a series of plateaus and regressions. This has been interpreted as some kind of "autoinhibition" of L-DOPA uptake and dopamine formation (Horst *et al.* 1974). If such a mechanism was involved at the dose levels

The Compliance Curve for the Flow Limiting Segments of the Airway I Model Studies

By

OLE FRID PEDERSEN and TAGE MORS NIELSEN

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Abstract

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By means of post-static tube airway compliance curves describing the cross-sectional area (A) as function of transmural pressure (P_{tm}) were constructed for several locations in the elastic airway of a mechanical model of the lung. From these curves local relations between elastic recoil pressure of the lung (P_{el}) and maximal expiratory flow (V_{max}) were calculated and compared with the experimentally determined P_{el} - V_{max} curve for the entire airway I—all parts in series. Theory and experiments showed that the lower the airway resistance of all the local P_{el} - V_{max} curves. This means that the maximal flow through the entire airway at a given P_{el} is determined by the segment of the airway having the smallest V_{max} . A is the maximal strength of a chain is determined by its weakest link. The relation between the critical transmural pressure (P_{tm}) and the corresponding cross-sectional area (A) was derived from the experimental P_{el} - V_{max} curve. This P_{tm} - A curve had a composed appearance, which was found to reflect parts of the different local P_{tm} - A curves and transitions between them because of movement of the flow limiting site within the airway. The P_{tm} - A curve depends on the elastic properties of the flow limiting segment, and the slope of this curve (dA/dP_{tm}) is the compliance of the flow limiting segment. Significant fractional pressure losses upstream from the site of flow limitation caused underestimation of both A and dA/dP_{tm} . but downstream pressure losses had no influence on the P_{tm} - A curve.

Key words: Mechanics of the expiratory airway compliance, maximum flow—static recoil curves, model experiments.

A functional relation has been shown to exist between transpulmonary pressure, lung inflation, and respiratory gas flow (Fry and Hyatt 1960). Graphically this relation can be described by a three-dimensional surface, on which the expiratory isovolume pressure-flow curves at least at the lower lung volumes present maxima. This means that increasing the transpulmonary pressure at a given lung volume, the flow will increase to a maximal value and then remain constant, or decrease slightly for further increase in the transpulmonary pressure.

During a forced vital capacity manoeuvre the transpulmonary pressure usually exceeds what is necessary for maximal expiratory flow during the lower 60-80% of the vital capacity

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$$\frac{dA}{dP_{tm}} = \frac{A}{2cV_{max}^2} \quad (1)$$

In this equation P_{el} is the elastic recoil pressure of the lungs. In case of significant frictional pressure loss due to the change in the cross-sectional area A , here the transmural pressure is P_{tm} , the equation is still valid if we substitute $P_{el} - P_{fr}$ for P_{el} , P_{fr} being these pressure losses. ζ is the flow velocity in the vessel lumen, constant depending on the density of the gas and the contraction coefficient (C_c) and by the equation $A_j = C_c A$, here A_j is the area of the jet passing through A (Streeter 1966). In air at ambient air at 25°C we get $0.603 (C_c)^2 \text{ g cm}^{-2} \text{ s}^2$ here V is measured in l s^{-1} , A in cm^2 and is pressure in cm H_2O (Pedersen and Nielsen 1976). dV_{max}/dP_{el} is the slope of the curve describing flow as function of P_{el} . dA/dP_{tm} is the slope of the airway compliance curve described by A as function of P_{tm} . The notation $()$ indicates that the parameter is valid where and when the flow is maximal in the airway. At $V = V_{max}$ we have that $P_{el} = P_{fr}$. The notation $()$ is therefore not necessary in this case. If V_{max} the measured $P_{el} - P_{fr}$ only if the tip of the pitot-static tube is at the flow limiting site. In following, P_{fr} indicates the closest measured approximation to P_{fr} .

Equation 2 states that it is possible to calculate A at any value of V_{max} . If the flow is turbulent and the curve describing ζ as function of P_{el} is known, because A is determined by V_{max} and the slope of the curve, dV_{max}/dP_{el} at the same V_{max} . The corresponding P_{tm} can next be calculated from equation 1 solved for P_{tm} , and ζ as V_{max} , and A substituted for V and A . Accordingly, one can transform the P_{el} -flow curve into P_{tm} - A curve. The relationship between P_{tm} and A is determined by the elastic properties of the flow limiting apparatus of the airway not necessarily located at fixed site.

Equation 3 states that one can calculate V_{max} at any value of A if the P_{tm} - A curve is known, because V_{max} is determined by A and the slope of the curve, dA/dP_{tm} is the same point. The corresponding value of P_{el} can next be calculated from equation 1 by substituting A , V_{max} and P_{tm} for A , V and P_{tm} . One can therefore also transform the P_{tm} - A curve into P_{el} - V_{max} curve.

In the previous paper it has been shown that it is possible to locate point in the collapsible airway of mechanical model of the lungs where measured relationship between P_{tm} and A , substituted into equation 2, gave values of V_{max} equal to measured values, at least during part of the hypothetical expiration.

In this study we intend to show that if frictional pressure losses can be neglected ($P_{fr} = 0$) then flow limitation takes place at the particular point in the curve y where the measured local P_{tm} - A curve, transformed into P_{el} - V_{max} curve by means of equations 3 and 1 for given P_{el} gives the smallest V_{max} of all such transformations along the collapsible airway.

By furthermore intend to show that the P_{tm} - A curve calculated from the relationship between P_{el} and V_{max} , back the same at all points in the airway is composed of fractions of different local P_{tm} - A curves, each contributing to flow limitation during part of the expiration, and transforms between these.

Finally, we intend to examine how this curve is affected by change in the resistance upstream and downstream from the flow limiting site in the airway.

As in the previous study we only examine the conditions in collapsible airway during forced expiration before and when flow is just maximal, whereas the conditions after the maximal flow has been reached are not the object of the study. In the present paper V_{max} always means just maximal flow.

Methods and Material

The experimental set-up has been described in detail in the previous paper (Pedersen and Nielsen 1976). Briefly, the lung model consisted of an air-tight transparent box containing one pair of spring-loaded bellows connected to the exterior of the box through an airway. This airway had a rigid upstream part, an elastic middle part, and a rigid downstream part. The elastic part was made of latex finger cuff with the top cut off, and formed into a 5 cm long converging-diverging segment with an internal diameter of 12 mm at the ends and 7 mm in the middle. At either end of the collapsible airway were slides with holes of various sizes to insert into the airway in order to vary the upstream and downstream resistances. A third slide with holes could be inserted for addition of external resistances downstream from the site, where lateral mouth pressures were measured. Lateral pressures could furthermore be measured in the bellows (the "alveoli"), and in the box (the "pleural" space).

(Hyatt 1961 Mead *et al* 1967 van de Woestijne and Zapletal 1970) Expiratory maximum effort flow volume (MEFV) curves will therefore in this interval present maximal, or near maximal flows. Analysis by Fry (1958) concluded that the maximal flows were determined by the characteristics of the flow and the physical properties of the intrathoracic pulmonary system. Therefore the MEFV curve was important for diagnostic purposes, as an abnormal curve reflected intrathoracic pulmonary abnormalities (Hyatt 1965).

The physical characteristics of the intrapulmonary system determining the maximal flow have therefore been a matter of interest (Mead *et al* 1967 Pride *et al* 1967 Fry 1968, Padoens *et al* 1972, Clément *et al* 1973 and Lambert and Wilson 1973). From these studies it can be concluded that the elastic properties of the airway (the airflow resistance in the peripheral part of the airway and the elastic recoil pressure of the lungs, closely related to the lung volume (Stead, Fry and Ebert 1952) are all important determinants of the maximal flow.

In humans the relations between maximal flow and lung volume on the one hand and static elastic recoil pressure and lung volume on the other can be obtained, and construction of maximum flow-static recoil (MFSR) curves is therefore possible (Mead *et al* 1967). Assuming that static and dynamic recoil pressures are equal, the resistance in the part of the airway upstream from the equal pressure point (EPP) can be determined from these curves (Mead *et al* 1967) or the resistance upstream from the flow limiting segment of the airway and the transmural pressure of the airway at the site of flow limitation can be determined if the latter can be regarded as a constant (Pride *et al* 1967). This is probably not the case (Pedersen and Nielsen 1976).

The purpose of the present investigation is to evaluate the MFSR curve, or rather the relationship between the dynamic elastic recoil pressure of the lungs and the maximal expiratory flow in terms of the relation between the transmural pressure of the airway where and when the flow is maximal and the corresponding cross-sectional area.

It is an extension of a previous work dealing with the critical transmural pressure of the airway (Pedersen and Nielsen 1976).

The theory will be briefly reviewed and evaluated in experiments with a mechanical model of the lungs.

Theory

It is assumed that the elastic properties of the flow limiting segments of the airway can be described by the elastic properties of a single airway behaving exactly the same way.

It is assumed that the flow is turbulent (see below), and the lateral pressure drop from the alveoli to a location in the airway with the cross-sectional area A is solely caused by convective acceleration of the gas molecules, but is a linear velocity of zero cm/s at the alveoli and large velocity when passing through.

It is furthermore assumed that a maximal expiratory flow occurs at any value of the elastic recoil pressure of the lungs when the transmural pressure at some point in the airway reaches critical value, which is less than the elastic recoil pressure.

In that case the following set of equations can be derived (Pedersen and Nielsen 1976)

$$P_{al} - \frac{1}{2} \rho V^2 = P_{tm}$$

$$\frac{dV}{dt} \max = \frac{A^{1/2}}{2cV_{ms}}$$

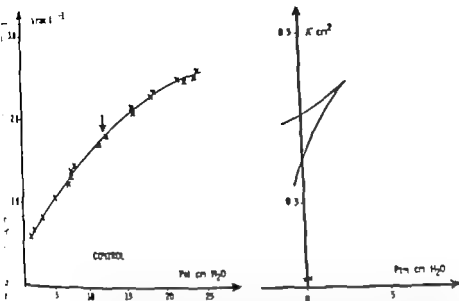


Fig. 1. Left: The relation between elastic recoil pressure (P_{el}) and maximal expiratory flow (V_{max}) in a system with different inertial tank pressures and/or different sizes of the external stenoses in the control system. Crosses are experimental points, and the curve through them is fitted by eye. Right: The derived relation between the critical transmural pressure (P_{tm}) and the corresponding cross-sectional area (A) (cf. (4) and (5)). During the expiration, proceed along the upper branch of the angle towards the top point corresponding to the arrow at the P_{el} - V_{max} curve and then descend along the lower branch.

collapsible airway. Both in the downstream part of the airway (at position A) and in the upstream part (at position C) the cross-sectional areas at given P_{tm} values are greater than flow measured closer to the mid point of the segment (at positions B_L and B_R). This is because the segment is converging-diverging.

In the previous paper it was shown that the part of the P_{tm} -A curve visualized in expiration with just maximal flow depended on P_{el} at full inflation of the bellows of the mechanical model. The same curve, however, could be obtained from the time course of P_{ca} , P_{tm} , and \dot{V} before flow during expiration reached maximal values, and in that case it could be extended towards larger P_{tm} values. The P_{tm} -A curves for positions III and B_{II} were in this way extended slightly by including points where $V < V_{max}$.

It appears from Fig. 2 that these two curves are slightly different. At large P_{tm} values the cross-sectional area is smallest at B_L , but at low P_{tm} it is smallest at B_{II} . The reason for this could be an inhomogeneity of the elastic airway which was not anticipated during construction.

The curves in Fig. 2 were transformed into the corresponding P_{el} - V_{max} curves (or $(P_{el}-P_{air})$ - V_{max} curves if $P_{air} \neq 0$, cf. above) by use of equations 3 and 1. These curves are shown in Fig. 3. They are the P_{el} - V_{max} curves we would get if the airway at the respective points in the elastic airway were indeed flow limiting and $P_{air} = 0$. It is seen that at high P_{el} the airway at position B_L has the smallest \dot{V}_{max} , whereas at low P_{el} it is the airway at

In order to determine the elastic properties of the collapsible segment in situ, the relationship between P_{tm} and A , a pitot-static tube was inserted into the airway. Simultaneous measurements of the pressure difference between the end hole and the side hole of this tube ($P_{ca} = P_{tot} - P_{stat}$), the transmural pressure ($P_{tm} = P_{stat}$ minus pleural pressure, P_{pl}) and the flow through the airway (V), made it possible to derive the cross-sectional area corresponding to the tip of the pitot-static tube (cf. Pedersen and Nielsen 1976). Then A could be plotted as a function of P_{tm} . By moving the tip of the pitot-static tube, such plots could be done for different points in the collapsible segment, representing the local P_{tm} - A relationships.

Forced expirations were produced after maximal inspiration by rendering the box pressure positive to a degree depending on the initial pressure in a pressure tank suddenly connected to the box. The initial tank pressure and the aperture of the externally applied stenosis would then determine the effect on the transpulmonary pressure with which the expiration would be performed.

During forced expirations with different initial tank pressures (10, 20, and 40 mmHg) lowest and highest externally applied stenosis (8 mm, 6 mm, 5 mm, and 4 mm in diameter), the time course of V , P_{tm} , P_{ca} , was recorded in one series of experiments and the time course of V and P_{el} in another otherwise identical series. On these curves just maximal flows were detected as described previously (Pedersen and Nielsen 1976) and corresponding values of V_{max} , P_{tm} , P_{ca} , and P_{el} were read on the curves. For a given position of the pitot-static tube a P_{tm} - A curve valid for $V = V_{max}$ and a P_{el} - V_{max} curve were constructed. Two positions were chosen for the tip of the pitot-static tube: position A was in the downstream part of the collapsible segment, positions B₁ in the middle, position B₂ a little above the middle, and position C in the upstream part of the collapsible segment.

By use of the 4 different P_{tm} - A curves, equations 3 and 1 ($C_e = 1.03$ for the converging part of the airway and $C_e = 0.77$ for the diverging part of the airway (Pedersen and Nielsen 1976), the corresponding P_{el} - V_{max} curves (or rather $(P_{el} - P_{sfr})$ - V_{max} curves, cf. above) were calculated. These curves corresponded to our theories the P_{el} - V_{max} relations we would obtain, if the segment with the given P_{tm} - A relation were indeed flow limited and $P_{sfr} = 0$. These calculated local curves were then compared with the experimental P_{el} - V_{max} curves for the entire airway to see if there were any concordance.

Some of the experiments were repeated after inserting stenosis upstream or downstream into the upstream part of the airway so that the diameter of the airway was reduced by approximately 50%. The purpose was to see whether a change in the upstream or downstream resistance would affect the relationship between P_{el} and V_{max} and the derived P_{tm} - A relationship.

Results

If the mechanical model should be in accordance with the theoretical model on which the derived equations were based, significant frictional pressure losses (P_{sfr}) between the alveoli and the cross-section A should be included in the equations by substituting $P_{el} - P_{sfr}$ for P_{el} . As shown in the appendix, section I, $P_{el} - P_{sfr}$ is equal to $P_{ca} + P_{tm}$ and could therefore be calculated from the tracings, and so could P_{sfr} for known values of P_{el} . With no stenosis inserted into this part of the airway P_{sfr} was usually small and could be neglected.

Fig. 1 (left part) shows the relation between P_{el} and V_{max} without insertion of any upstream or downstream stenosis into the airway. The crosses are experimental points, and the curve through them is drawn by eye. The curve is transformed into the corresponding P_{tm} - A curve by use of the equations 2 and 1. The transformed curve is shown in the right part of the figure. At the highest value of V_{max} (and P_{el}) the relation between P_{tm} and A gives a point to the far left on the upper branch of the transformed curve. As the expiration proceeds, P_{tm} and A increase and we ascend along the upper branch towards the top point of the angle (corresponding to the arrow at the P_{el} - V_{max} curve). As the flow decreases further we move back again along the lower branch of the angle, with decreasing values of P_{tm} and A .

Fig. 2 shows the measured relationships between P_{tm} and A at the four points in the

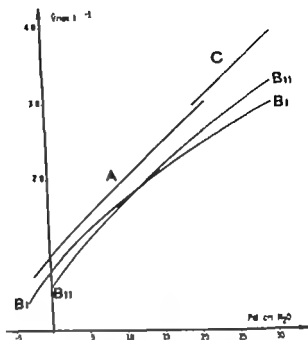


Fig. 3 Relations between elastic recoil pressure (P_{el}) and maximal respiratory flow (V_{max}) calculated from the corresponding curves in Fig. 2 by use of equations 3 and 1.

To see if this were the case in the present experiments, the experimental points of Fig. 1 upper part, were plotted in a diagram showing the lower borderlines of the curves in Fig. 3. The result is shown in Fig. 4 (left part). The curve to the right of the arrow is part of B_{II} and the curve to the left is part of B_I in Fig. 3. The experimental points and the theoretical curves appear indeed to agree fairly well, although the experimental points are placed slightly to the right of these curves. This may be due to $P_{sfr} = 0$, as P_{sfr} is the horizontal distance between a $(P_{el} - P_{sfr}) - V_{max}$ curve and a $P_{el} - V_{max}$ curve.

The Ptm-A curve has a course that cannot be immediately explained by the different local Ptm-A curves (compare Fig. 1 right part, with Fig. 2). The descending portion is in harmony with part of B_{II} , but the ascending portion has apparently no correlate. If we assume that the composed theoretical curve in Fig. 4 left part, is indeed the actual $P_{el} - V_{max}$ curve, then the corresponding Ptm-A curve must be made up by parts of the curves B_I and B_{II} in Fig. 2, because the theoretical curve was derived from these two curves. The question about which parts of the curves are involved, can be solved by use of equations 2 and 1 with substituted V_{max} and dV_{max}/dP_{el} valid for the end points of the curve sections B_I and B_{II} in Fig. 4 left part. The right part of Fig. 4 shows sections of the curves B_I and B_{II} from Fig. 2, but only the full-line fragments of these curves are involved in flow limitation. During the hypothetical expiration where $V = V_{max}$ throughout, we proceed from right to left on curve B_I . Reaching the arrow at B_{II} , the site of flow limitation suddenly shifts a little further upstream, where the curve B_{II} is valid. There is a discontinuous jump between the two arrows both corresponding to the arrow at the $P_{el} - V_{max}$ curve. After this jump we move down curve B_{II} as the expiration proceeds. The discontinuous jump is due

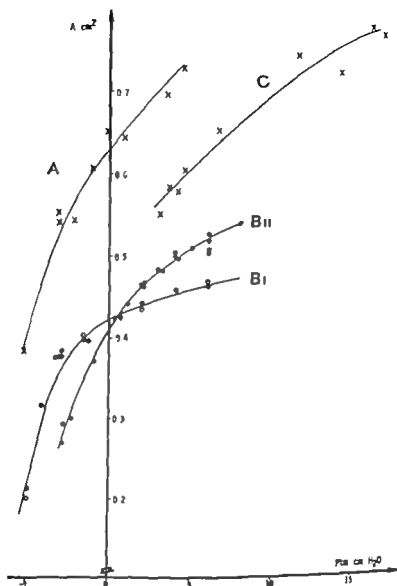


Fig. 2. Measured relations between transmurial pressure (P_{tm}) and cross-sectional area (A) at 4 positions of the pitot-static tube in the collapsible segment. Position A is in the downstream part of the segment. Position B_I is the middle, position B_{II} is little above the middle, and position C is the upstream part of the segment. The crosses, circles, and filled-out circles are experimental points. The curves through them are fitted by eye.

position B_{II}. The change of P_{el} from positive to negative values at low V_{max} means that the elastic forces tend to expand, rather than constrict the "lung". This is only possible, if the airway can resist a negative transmurial pressure without collapsing.

As the flow is identical in all parts of the airway (flow caused by airway collapse is assumed to be negligible) the maximal flow must be determined by the part of the airway with the smallest V_{max} , just as the strength of a chain is determined by its weakest link. The P_{el} - V_{max} curve for all parts in series must therefore be the lower borderline of all curves each describing local flow maxima at different points along the elastic airway.

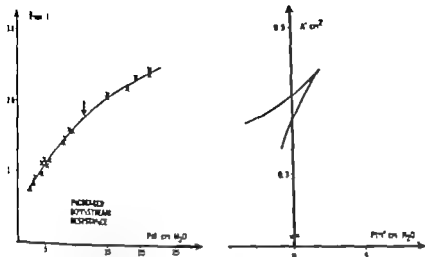


Fig. 5. Left: The relation between transalveolar pressure (P_{TM}) and maximal expiratory flow rate (\dot{V}_{max}) in experiments with increased downstream resistance. Right: The derived relation between the critical transmural pressure (P_{Tr}) and the corresponding cross-sectional area (A). Compare with the control situation in Fig. 1.

curves in Fig. 1 and 5. The position of the latter is not changed, but it is shorter corresponding only to the lower half of these curves. By means of equations 2 and 1 the curves are transformed into the corresponding P_{TM} - A curves. In the latter case P_{Tr} - P_{Tr} was substituted for P_{Tr} . The result is shown in the right part of the figure. If P_{Tr} - P_{Tr} is substituted for P_{Tr} , the P_{TM} - A curve has a course which is similar to the descending branch of the P_{TM} - A curves in Fig. 1 and 5. If P_{Tr} is neglected and P_{Tr} is substituted into the equations 2 and 1, A is underestimated at all P_{Tr} as expected (cf. above). It is furthermore seen that the slope of the curve, i.e. the airway compliance, is underestimated. Therefore, the airway appears to be more stiff than it actually is.

Besides, the curve is shifted slightly to the left, which means that P_{TM} is underestimated by neglecting P_{Tr} . P_{Tr} was measured as the horizontal distance between the curves in the left part of the figure, and $\log P_{Tr}$ plotted as a function of $\log \dot{V}_{max}$ in Fig. 7. The best fitting straight line has the slope 2.58, which agrees with the finding that P_{Tr} is underestimated (cf. appendix, section II).

Discussion

In these experiments we measured the relations between transmural pressure and cross-sectional area at 4 positions in a converging-diverging elastic airway of a mechanical model of the lung. Four local P_{TM} - A curves were drawn and transformed into corresponding P_{Tr} - \dot{V}_{max} curves, which were compared with the measured, actual P_{Tr} - \dot{V}_{max} curve, obtained with the tip of the piezo-static tube placed little above the middle in the airway (at position B_{III}). This comparison is justified only if frictional pressure losses can be neglected as in most of these experiments and if the local P_{TM} - A curves are not influenced by the

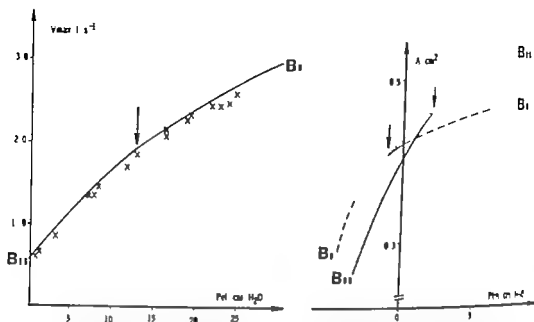


Fig. 4. *Left* The experimental points of Fig. 1 left part, plotted in a diagram showing the lower border line of the curves in Fig. 3. This is composed of the curve sections B_I and B_{II} on each side of the arrow (marked by the arrow). *Right* Here B_I and B_{II} are parts of the corresponding curves in Fig. 2 but only the B_I -line fragments are derived from the curve sections above. During the expiration we proceed down the full-line fragment of B_I . Reaching the arrow we jump to B_{II} and continue down this curve. The jump between the two arrows both corresponding to the one arrow at the P_{el} - V_{max} curve above is indicated by the dotted curve.

to the fact that the curve sections B_I and B_{II} in Fig. 4 left part, form an obtuse angle at the arrow. The best fitting curve (Fig. 1 left part) has a smooth course, and therefore the P_{tm} - A curve (Fig. 1 right part) shows no discontinuity. Its ascending portion may therefore reflect the upstream motion of the flow limiting site (i.e. motion from B_I to B_{II}) as indicated by the dotted curve between the two arrows in the right part of Fig. 4.

As downstream pressure drops do not appear in equations 1, 2 and 3 no change in these relations were expected after insertion of a downstream stenosis. This is confirmed by Fig. 5 which shows the effect of an increased downstream resistance on the P_{el} - V_{max} curve (left part of the figure) and on the derived P_{tm} - A curve (right part of the figure). Compared with Fig. 1 there are no marked differences. But no differences were expected either.

Insertion of an upstream stenosis increased P_{sfr} which could not be considered negligible any more. Therefore P_{el} - P_{sfr} should be substituted for P_{el} in equations 1 and 2, if they should still be valid. If we neglect the influence of P_{sfr} which presumably increases with V_{max} , then A and dA/dP_{tm} will both be underestimated as shown in the appendix, section II. It is here furthermore shown that P_{tm} may be overestimated or underestimated depending on the relation between V_{max} and P_{sfr} .

To check this hypothesis, P_{tm} - A curves were constructed by substitution of both P_{el} and P_{el} - P_{sfr} into equations 1 and 2 in the experiments with increased upstream resistance.

Fig. 6 shows the effects of an increased upstream resistance. In the left part of the figure are shown two curves. One is the P_{el} - V_{max} curve, and the other gives the relation between P_{el} - P_{sfr} and V_{max} . The former is shifted to the right compared with the corresponding

lower horizontal of the four curves. Stepwise withdrawal of the tube from B₁₁ in B₇ and prior to A will affect the P_{tm}-A relations at these positions in a similar way but it is difficult to tell how this will influence the flow limiting properties of the airway.

The effect of complete removal of the pitot-static tube from the collapsible part of the airway was a slight increase of V_{max} at all P_{el} values, and a corresponding increase of A at all P_{el} values, with actually no change of P_{tm}. The increase of A was about 0.07 cm² and this equaled almost exactly the cross-sectional area of the pitot-static tube, which determined the part of the cross-sectional area available for airflow *Le* around the tube. The fact that this increase of A was the only effect of removal of the pitot-static tube indicates that the behaviour of the collapsible segment was not influenced by the presence of the tube.

The concordance between the measured points and the calculated P_{el}-V_{max} curve (fracture giving the smallest V_{max} at a given P_{el} (cf. Fig. 4 left part) indicates that the segment of the airway with the smallest V_{max} at a given P_{el} is the flow limiting segment, and we find no evidence that the presence of the pitot-static tube interferes with this conclusion.

In the present experiments it was not the same segment that was flow limiting at all V_{max} values. Therefore, the P_{tm}-A curve derived from the measured P_{el}-V_{max} curve was not identical with any particular local P_{tm}-A curve, but had a composed appearance with an ascending and a descending branch (cf. Fig. 1 right part). The further analysis indicated that the ascending branch coincided with motion of the flow limiting site from one part of the segment to another with a different P_{tm}-A relation. The descending part, however, corresponded closely to the P_{tm}-A relation at this other part of the segment.

The factors determining which airway segment is flow limiting may be evaluated. Both the cross-sectional area A and the airway compliance dA/dP_{tm} at the same A are determined (cf. equation 3). Fig. 4 right part, shows that the cross-sectional area at the left arrow is smaller than that at the right arrow. The motion of the flow limiting site from B₇ to B₁₁ along the dotted curve involves that flow limitation does not take place at the smallest cross section any more.

If the site of flow limitation moves to a segment with a larger cross-sectional area, the new V_{max} can only be achieved if also the compliance of the airway dA/dP_{tm} is larger at the new site. This is stated in equation 3, and is illustrated in Fig. 4 right part, where the points corresponding to the two arrows both satisfy equation 3 for the same V_{max} (1.9 l/s).

The present experiments were intended to elucidate the conditions at just maximal flows. We therefore only described conditions which may be assumed to correspond with the curve through the points of maximal flow on the three-dimensional surface of Fry and Hyatt (1958) described by way of introduction. Flows at larger transpulmonary pressures than necessary for just maximal flow may still be maximal in the sense that they are determined by equation 3, solved for V_{max}, but the local P_{tm}-A curves may then be changed, due to downstream compression of the collapsible airway (Pedersen and Nielsen 1976). An elucidation of these conditions is beyond the scope of the present study.

As the ultimate purpose of the present investigation is to explain the MFSR curves obtained in experiments with humans, we will now discuss the results from this point of view. In the human peripheral airway frictional pressure losses may possibly not be neglected.

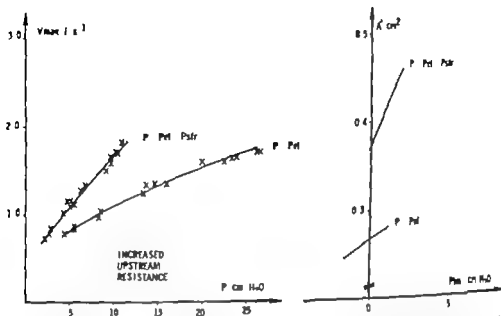


Fig. 6. *Left* The relation between elastic recoil pressure (P_{el}) and maximal expiratory flow rate (V_{max}) as the pressure loss with increased upstream resistance. V_{max} is also shown as a function of the elastic recoil pressure as the pressure loss between the bellows and the tip of the pitot-static tube at position B_{II} ($P_{el}-P_{el}$). The crosses are experimental points and the curves through them are fitted by eye. *Right* The relation between the critical transmural pressure (P_{tmr}) and the corresponding cross-sectional area (A) determined from the two curves above (cf. text). Compare with Fig. 1 and 3.

presence of the pitot-static tube by means of which they were measured and which had to be moved from one position to another between the experiments.

Curve C in Fig. 2 was measured with the tip of the pitot-static tube at position C. I cannot tell exactly what the $P_{tm}-A$ relation at C will be, when the tube is withdrawn to position B_{II} which is our point of reference, but the cross-sectional area available for airflow at position C will increase as the area occupied by the tube is added, whereas the cross-sectional areas at the further downstream positions A, B_I and B_{II} are expected to remain unchanged because the tip of the tube has approximately the same diameter as the trachea itself. Curve C in Fig. 2 would therefore be displaced upwards, and so would the $P_{el}-V_{max}$ curve calculated from it. Such a displacement of curve C in Fig. 3 would not change

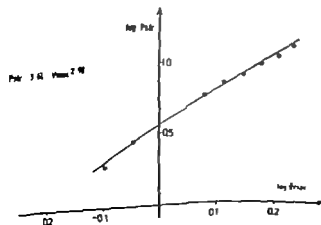


Fig. 7 The logarithm to P_{str} versus V_{max} flow rate. It is the horizontal distance between the two curves in the upper part of Fig. 6, plotted as a function of $\log V_{max}$. The best fitting line through the points is given by the line in the figure.

the borderline of the four curves. Stepwise withdrawal of the tube from B₁₁ to B₁ and later to A will affect the P_{tm}-A relations at these positions in a similar way but it is difficult to tell how this will influence the flow limiting properties of the airway.

The effect of complete removal of the pitot-static tube from the collapsible part of the airway was a slight increase of V_{max} at all P_{el} values, and a corresponding increase of A at all P_{tm} values, with actually no change of P_{tm}. The increase of A was about 0.07 cm² and this equalled almost exactly the cross-sectional area of the pitot-static tube, which determined the part of the cross-sectional area available for airflow *Le* around the tube. The fact that this increase of A was the only effect of removal of the pitot-static tube indicates that the behaviour of the collapsible segment was not influenced by the presence of the tube.

The concordance between the measured points and the calculated P_{el}-V_{max} curve (fractiles giving the smallest V_{max} at a given P_{el} (cf. Fig. 4, left part) indicates that the segment of the airway with the smallest V_{max} at a given P_{el}, is the flow limiting segment, and we find no evidence that the presence of the pitot-static tube interferes with this conclusion. In the present experiments it was not the same segment that was flow limiting at all V_{max} values. Therefore, the P_{tm}-A curve derived from the measured P_{el}-V_{max} curve was not identical with any particular local P_{tm}-A curve, but had a composed appearance with an ascending and a descending branch (cf. Fig. 1 right part). The further analysis indicated that the ascending branch coincided with motion of the flow limiting site from one part of the segment to another with a different P_{tm}-A relation. The descending part, however, corresponded closely to the P_{tm}-A relation at this other part of the segment.

The factors determining which airway segment is flow limiting may be evaluated. Both the cross-sectional area A and the airway compliance dA/dP_{tm} at the same A are determinants (cf. equation 3). Fig. 4 right part, shows that the cross-sectional area at the left arrow is smaller than that at the right arrow. The motion of the flow limiting site from B₁₁ to B₁ along the dotted curve involves that flow limitation does not take place at the smallest cross section any more.

If the site of flow limitation moves to a segment with a larger cross-sectional area, the maximal V_{max} can only be achieved if also the compliance of the airway dA/dP_{tm} is larger at the new site. This is stated in equation 3 and is illustrated in Fig. 4 right part, where the points corresponding to the two arrows both satisfy equation 3 for the same V_{max} (-1.9 l/s).

The present experiments were intended to elucidate the conditions at just maximal flows. We therefore only described conditions which may be assumed to correspond with the curve through the points of maximal flow on the three-dimensional surface of Fry and Hyatt (1962) described by way of introduction. Flows at larger transpulmonary pressures than necessary for just maximal flow may still be maximal in the sense that they are determined by equation 3 solved for V_{max}, but the local P_{tm}-A curves may then be changed, due to downstream compression of the collapsible airway (Pedersen and Nielsen 1976). An elucidation of these conditions is beyond the scope of the present study.

As the ultimate purpose of the present investigation is to explain the MFR curves obtained in experiments with humans, we will now discuss the results from this point of view. In the human peripheral airway frictional pressure losses may possibly not be neglected.

Therefore we examined what the curves would look like if upstream frictional pressure losses (P_{sfr}) were neglected after insertion of an upstream stenosis. We found that the cross sectional area of the flow limiting segment was underestimated, and that this was also the case with the compliance of the flow limiting segment, i.e. the slope of the $P_{tm}-A$ curve. The critical transmural pressure, however, was not much changed because P_{sfr} varied almost proportional to V_{max} squared.

In the model, the upstream stenosis was independent of the lung volume, and therefore had a constant cross-sectional area. In that case P_{sfr} should vary with V_{max} . This is not far from what we found. In humans, the linear dimensions of the peripheral airways vary with approximately the cube root of the thoracic gas volume (Hughes *et al.* 1977). During the expiration the dimensions of these airways therefore decrease, and the exponent n in the equation $P_{sfr} = k_{sfr} V_{max}^n$ will even in case of complete turbulent flow probably be less than 2 (Pedersen 1973 p. 102 ff.). Neglecting of P_{sfr} therefore, will probably cause P_{tm} to be overestimated (cf. appendix, section II).

The unique relation between maximal expiratory flow and elastic recoil pressure or lung volume has been challenged by several authors. As pointed out by Mead *et al.* (1967) the maximal flow will depend also on time in case of unequal ventilation of different lung units. In a monalveolar lung model the maximal flow measured at the mouth will be time dependent too due to flow from the collapsing airway (Clément *et al.* 1973 b) and airway wall resistance or inertia (Clément *et al.* 1974).

In the mechanical model we assumed that time factors could be neglected. This was probably true for several reasons. Firstly it was a monalveolar model, and no inequality of ventilation could exist. Secondly the volume of the collapsible airway was very small, and airway flow therefore minimal. Airway flow is expected to be largest when the rate of change of transpulmonary pressure is large (Clément *et al.* 1973 b). For these reasons airway flow could only be expected to contribute at high V_{max} in the present experiments due to this gradient otherwise being small. Finally inertia and resistance of the wall of the collapsible segment could probably be neglected, as this was very thin.

By application to the lungs of the theories evaluated here, these factors may not be negligible and must therefore be included in the considerations.

Appendix

Section I

As defined in the text,

$$P_{ca} = P_{tot} - P_{lat} \quad (1.1)$$

$$P_{tm} = P_{lat} - P_{pl} \quad (1.2)$$

By addition,

$$P_{ca} + P_{tm} = P_{tot} - P_{pl} \quad (1.3)$$

As P_{sfr} is the difference between the pressure inside the bellows (P_{alv}) and the end-hole pressure of the pitot-static tube,

$$P_{sfr} = P_{alv} - P_{tot} \quad (1.4)$$

Substituting $P_{alv} = P_{pl} + P_{pl}$, and rearranging,

$$P_{tot} - P_{pl} = P_{el} + P_{sfr} \quad (1.5)$$

Adding (I-3) and (I-5),

$$P_{01} + P_{02} = P_{01} - P_{02} \quad (I-4)$$

hence if

there is significant upstream frictional pressure loss, P_{01} then $P_{01} - P_{02}$ should be substituted for P_{01} in equation 1, the arc, and get:

$$P_{01} - P_{02} = \frac{1}{A} \dot{V}^2 + P_{02} \quad (II-1)$$

If we neglect P_{02} , equation II-1 may be written

$$P_{01} = \frac{1}{A^2} \dot{V}^2 + P_{02} \quad (II-2)$$

where A_0 and P_{02} are not necessarily equal to A and P_{01} .

For $\dot{V} = \dot{V}_{max}$ we get from equation 2 in the text

$$\frac{dP_{01}}{d\dot{V}_{max}} = \frac{dP_{02}}{d\dot{V}_{max}} = \frac{2c\dot{V}_{max}}{A^3} \quad (II-3)$$

In the first case, and

$$\frac{dP_{01}}{d\dot{V}_{max}} = \frac{2c\dot{V}_{max}}{A} \quad (II-4)$$

In second case,

From equations II-3 and II-4 see that if $dP_{02}/d\dot{V}_{max} = 0$, then $A = A_0$ and $dP_{01}/d\dot{V}_{max} = 0$, then $A_0 = A$. This means that neglecting of P_{02} may cause A to be underestimated.

For $\dot{V} = \dot{V}_{max}$ we get from equation 3 in the text

$$\frac{dA}{dP_{01}} = \frac{A}{2c\dot{V}_{max}^2} \quad (II-5)$$

and if P_{02} neglected

$$\frac{dA}{dP_{02}} = \frac{A_0}{2c\dot{V}_{max}^2} \quad (II-6)$$

As seen above, neglecting of P_{02} in case $A_0 < A$ if $dP_{02}/d\dot{V}_{max} = 0$. Because both A and $A_0 = 0$,

$$\frac{dA_0}{dP_{02}} < \frac{dA}{dP_{01}} \quad (II-7)$$

which means that the compliance of the airway may be underestimated at given \dot{V}_{max} . Let the airway may appear more stiff than actually is if P_{02} is neglected.

Subst. II-1 for P_{01} and substituting $\dot{V}^2/A^2 = c\dot{V}_{max}^2/A^2 = \dot{V}_{max}(dP_{01}/d\dot{V}_{max} - dP_{02}/d\dot{V}_{max})/2$ from II-3, we get for $\dot{V} = \dot{V}_{max}$

$$P_{01} - P_{02} = P_{01} - P_{02} = \frac{\dot{V}_{max}}{2} \left(\frac{dP_{01}}{d\dot{V}_{max}} - \frac{dP_{02}}{d\dot{V}_{max}} \right)$$

by rearranging,

$$P_{01} + P_{02} = \frac{\dot{V}_{max}}{2} \left(\frac{dP_{02}}{d\dot{V}_{max}} \right) + P_{01} - \frac{\dot{V}_{max}}{2} \left(\frac{dP_{01}}{d\dot{V}_{max}} \right) \quad (II-8)$$

Subst. II-2 for P_{02} and substituting $c\dot{V}^2/A^2 = \dot{V}_{max}^2/A^2 = \dot{V}_{max}(dP_{01}/d\dot{V}_{max})/2$ from II-4

get finally

$$P_{01} = P_{01} - \frac{\dot{V}_{max}}{2} \left(\frac{dP_{01}}{d\dot{V}_{max}} \right) \quad (II-9)$$

From equations II-8 and II-9 we see that

$$P_{tm} = P_{tm} + P_{str} = \frac{V_{max}}{2} \left(\frac{dP_{str}}{dV_{max}} \right) \quad (1)$$

If $P_{str} = k_{str} V_{max}^n$ where k_{str} and n are arbitrary constants, then

$$P_{tm0} = P_{tm} + \left(1 - \frac{n}{2} \right) k_{str} V_{max} \quad (2)$$

For $n < 2$ and $k_{str} > 0$

$P_{tm} > P_{tm0}$ i.e. neglect of P_{str} causes P_{tm} to be overestimated.

For $n = 2$ and $k_{str} > 0$

$P_{tm0} = P_{tm}$ i.e. neglect of P_{str} does not invalidate the determination of P_{tm} .

For $n > 2$ and $k_{str} > 0$

$P_{tm} < P_{tm0}$ i.e. neglect of P_{str} causes P_{tm} to be underestimated.

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Effect of the Polyene Antibiotic Filipin on the Permeability of the Inward- and the Outward-facing Membranes of the Isolated Frog Skin (*Rana temporaria*)

By

ROBERT NIELSEN

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Abstract

NIELSEN, R. Effect of the polyene antibiotic filipin on the permeability of the inward- and the outward-facing membranes of the isolated frog skin (*Rana temporaria*). Acta physiol. scand. 1977 99 399-411

The effect of the polyene antibiotic filipin on the electrical properties and the passive permeability of the frog skin was investigated. The addition of filipin to the outside bathing solution has the following effects: 1. it results in a drastic reduction in the transepithelial resistance and potential, 2. it causes 10-20% increase in the passive transepithelial chloride, sodium and sucrose flux, 3. it results in the formation of an amiloride insensitive sodium pathway in the outward facing membrane, 4. it results in an activated transport of potassium, 5. it results in a highly significant swelling of all the cells in the epithelium. The addition of filipin to the inside bathing solution has the following effects: 1. it results in an activation of the active sodium transport, 2. it causes a slight increase in the passive transepithelial chloride and sodium permeabilities but has no effect on the sucrose permeability, 3. it has no effect on the amiloride insensitive part of the short-circuit current, 4. it has no effect on the volume of the cells in the epithelium. It is suggested that the addition of filipin to the outside bathing solution increases the direct sodium flow from cell to cell or neighbor layers. Furthermore these experiments indicate that the outward facing membrane of the isolated frog skin has a high cholesterol content as compared with the cholesterol content of the inward facing membrane.

The polyene antibiotics are characterized by a large polyhydroxy lactone ring of 23-43 atoms with 4 to 7 conjugated double bonds in the ring. The polyene antibiotics filipin and rapamycin II mediate changes in the permeability of bilayer membranes, liposomes, erythrocytes, fungi and intestinal mucosal cells from chicks (for ref. see Norman *et al.* 1972).

In sodium transporting epithelia, such as the frog skin and the toad bladder the addition of amphotericin B to the outside (the mucosal side) bathing solution of the epithelia resulted in an increase in the passive chloride and urea permeabilities, whereas the effect on the active sodium transport was variable (Lichtenstein and Leaf 1965, Flinn 1968, Nielsen 1971). The addition of amphotericin B to the inside of the frog skin and the serosal side of the toad bladder had no effect on the active sodium transport (Lichtenstein and Leaf 1965).

Finn 1968 Nielsen 1971) On the basis of these and other experiments Lichtenstein and Le (1965) concluded that amphotericin B increased the permeability of a dense diffusion barrier near the mucosal surface to small solutes, whereas Finn (1968) concluded that amphotericin B increased the passive movement of sodium and small solutes across the membrane facing the medium to which the drug was added.

In a previous paper I have suggested a model to explain the changes in the sodium transport during the aldosterone induced moulting (Nielsen 1972, 1973) This model was partially based on the assumption that addition of filipin or amphotericin B to the outside bathing solution resulted in an increase in the passive transepithelial sodium flux.

In the present studies it is shown that addition of filipin to the outside bathing solution results in a 10–20 times increase in the passive transepithelial sodium, chloride and sucrose fluxes. Furthermore the addition of filipin to the inside bathing solution results in an increase in the active sodium transport but has nearly no effect on the passive transepithelial sodium chloride and sucrose fluxes.

Methods

The experiments were performed on male and female frogs (*Rana temporaria*). The frogs were kept initially immersed in tap water at about 4°C. The skins were dissected from pithed animals and divided into two laterally symmetrical pieces. The skins were mounted in perspex chambers and bathed in stirred Ringer solution (Na^+ 115.0 mM, K^+ 2.5 mM, Ca^{2+} 1.0 mM, HCO_3^- 2.4 mM, Cl^- 117.1 mM, pH = 8.2).

The short-circuit experiments were performed according to the method of Ussing and Zerahn (1957) using an automatic voltage clamp apparatus programmed to disconnect the short-circuit current every five minutes, thus allowing the potential to be measured for 60 s. The fluxes are in some experiments expressed in terms of permeability coefficients, calculated by adding the isotope to the medium bathing one side and measuring its rate of appearance on the other side (Andersen and Ussing 1957).

$$P = (a_2 V - a_1 V) / A \cdot a_2 (t_2 - t_1)$$

a_1 and a_2 designate the radioactivity originating from side 2 in one ml of solution from side 1 between t_1 and t_2 , respectively. $V = dV$ the corresponding volumes of the solution on side 1. A the area of membrane. a_2 the mean activity in one ml of solution from side 2 during the period from t_1 to t_2 . Filipin Lot 5-5956 was a gift from the Upjohn Company, Kalamazoo, Michigan, U.S.A.

Results

The effect of filipin on the short-circuit current (SCC) and transepithelial potential (PD)
Addition of filipin to the outside bathing solution Fig. 1 A shows the effect of adding 5 μ M filipin to the medium bathing the outside of the isolated frog skin. Fig. 1 B shows the control skin half 10–40 min after the addition of 5 μ M filipin the PD and resistance started to decrease. Higher concentrations of filipin (50 μ M) caused the decrease in PD and resistance to start 2–3 min after the addition of the filipin. 0.5 μ M had no effect on the PD and resistance. 5 μ M filipin caused a 40–75% reduction in the PD. 50 μ M filipin caused a 75–90% reduction in the PD. The effect of filipin on the SCC was variable. In about half of the experiments filipin had no effect on the SCC or caused 10–50% reduction in the SCC. In the remaining experiments filipin caused 10–100% activation of the SCC. This activation of the SCC lasted 1–2 h.

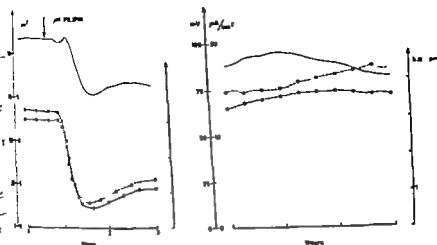


Fig. 1 A and B. Addition of filipin to the outside bathing solution. Effect of filipin on the short-circuit current and potential across the frog skin. A. At the arrow filipin was added to the outside to give a concentration of $5 \mu\text{M}$. B. Control (solid line). — short-circuit current ($\mu\text{A}/\text{cm}^2$); $\circ-\circ$ potential (mV). $t=0$ assumes (Kohler, 1967).

Kinsky (1963) has shown that $3 \mu\text{M}$ filipin caused complete hemolysis of rat erythrocytes, while $1 \mu\text{M}$ no hemolysis occurred. Van Zutphen *et al.* (1971) have shown that $0.4 \mu\text{M}$ filipin caused about 95% reduction in the resistance of lecithin cholesterol (1/1) bilayer membranes. In the presence of $0.4 \mu\text{M}$ filipin the lecithin cholesterol bilayer membrane was stable; in the presence of $4 \mu\text{M}$ filipin the survival time of the membrane was about 6 min, and in the presence of $40 \mu\text{M}$ the survival time of the membrane was less than 1 min (Van Zutphen *et al.* 1971). Thus the concentration of filipin that induced a reduction in the PD and the resistance across the isolated frog skin, hemolysis of rat erythrocytes, and a decrease in the survival time of bilayer membranes was the same. Therefore the decrease in PD and resistance in the isolated frog skin after the addition of filipin to the outside may be due to partial disintegration of the outward-facing membrane.

Addition of filipin to the inside bathing solution. Addition of $5 \mu\text{M}$ filipin to the inside had no effect on the PD and the SCC. About 5 min after the addition of $50 \mu\text{M}$ filipin the SCC and to a lesser degree the PD increased (Fig. 2 A). The increase in SCC was 10–100% of the control level. In half the expts. the current response to filipin addition was triphasic, the first maximum in SCC occurring 20–45 min after addition of filipin thereafter the SCC started to decline to about the control level. After this decrease the SCC increased again and reached its second maximum after about 2 h of incubation.

The effect of filipin on sodium flux

To ascertain whether the increase in the SCC after addition of filipin to the inside of the frog skin was due to an increase in the active sodium flux, a series of double labelling expts. were performed. The net sodium flux and the SCC were identical both in the control period and in the two periods after the addition of filipin (Table I). Thus, the addition of filipin to

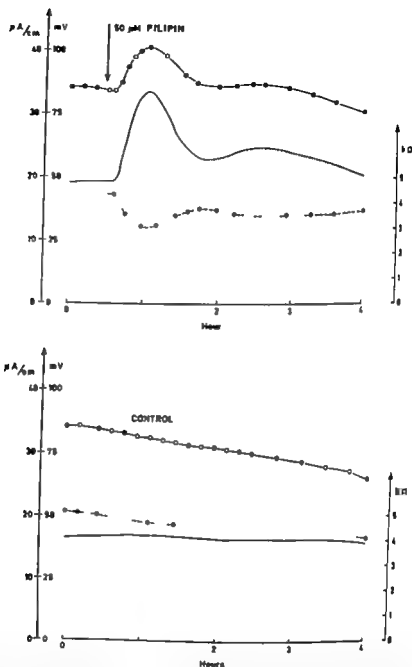


Fig. 2 A and B. Addition of filipin to the inside bathing solution. Effect of filipin on the current and potential across the frog skin. A: At the arrow filipin was added to the inside bathing solution of 50 μM . B: Control skin half. — short-circuit current ($\mu\text{A}/\text{cm}^2$) O—O potential (mV) O—O resistance (Kohm cm^2)

the inside bathing solution of the frog skin results in an activation of the active transport.

Effect on passive fluxes

To evaluate the effect of filipin on the permeability of the frog skin to substances, that of which can be accounted for by passive forces, transepithelial sucrose and (

TABLE I The net sodium flux and short-circuit current across the frog skin before and after the addition of filipin ($50 \mu\text{M}$) to the inside of the frog skin.

Period	Influx	Outflux	Net Flux	SCC	
			$\mu\text{eq}/\text{cm}^2/\text{h}$		
Control	1.16	0.03	1.11	1.12	$0.60 > p 0.50$
Filipin 1 h.	1.33	0.06	1.27	1.30	$0.60 > p 0.50$
Filipin 2 h.	1.26	0.04	1.22	1.19	$0.50 > p 0.40$

The skin was measured by means of Na-22 and the outflux simultaneously with Na-24. The SCC was measured and recorded automatically. The mean results from 7 expts. are presented. Each period was 1 h.

and outflux were determined. The sodium outflux (which is passive) was also measured. Table II shows that the addition of filipin to the outside bathing medium, (the skins were mounted in Ringer's solution containing 1 mM sucrose) results in large and sustained increases in the influx and outflux of sucrose, whereas the addition of filipin to the inside had no effect on these fluxes. The addition of filipin to the outside bathing medium results in a large and sustained increase in the chloride in- and outflux, and in the sodium outflux (Table III). Furthermore there was no significant difference between the chloride in- and outflux. The addition of filipin to the inside bathing medium had only a small effect on the chloride in- and outflux and on the sodium outflux (Table IV). Fig. 3 shows a series of experiments in which the sodium outflux is plotted against the chloride outflux. The outfluxes of sodium and chloride were measured simultaneously on the same skin after the addition of filipin to the outside bathing medium. The slope of the line drawn in Fig. 3 is equal to the ratio of the free solution mobilities of sodium and chloride *i.e.* 0.66. The experimental points fit the theoretical line. This indicates that the addition of filipin to the outside bathing medium results in the formation of a nonspecific transepithelial pathway.

Previous data (Nielsen 1971) have shown that the addition of $50 \mu\text{M}$ amphotericin B to the outside bathing solution results in a 5 times increase in the sodium outflux and a 5 times increase in in- and outflux of chloride and urea. The increases in the chloride and urea in- and outfluxes are essentially the same in both directions. The addition of amphotericin

TABLE II Effect of $50 \mu\text{M}$ filipin on the permeability of the frog skin to sucrose.

Period	Control \pm S.E. A-B	Filipin \pm S.E. B-C	C-D	D-E
Sucrose on outside	0.87 ± 0.24	7.76 ± 0.54	10.6 ± 0.6	13.7 ± 1.7
Sucrose on inside	0.61 ± 0.07	0.57 ± 0.11	0.59 ± 0.10	0.70 ± 0.09

In each expt. following 20 min of equilibration for the isotope the permeability across the tissue was determined for 1 h control period (period A-B). Filipin was then added to the outside or the inside bathing medium to give a concentration of $50 \mu\text{M}$. After the addition of filipin solution, the permeability was determined for 3 further 1 h periods (periods B-C, C-D and D-E). Both the sucrose in- and outflux were measured in these expts. The results were essentially the same for fluxes in either direction. All values are the mean \pm S.E. of 6 expts.

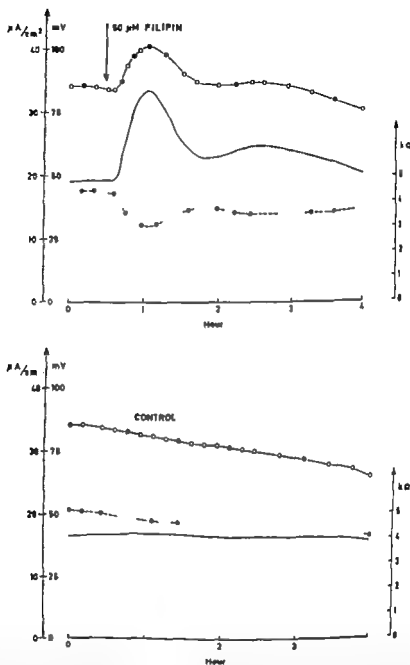


Fig. 2 A and B Addition of filipin to the inside bathing solution. Effect of filipin on the short-current and potential across the frog skin. A. At the arrow filipin was added to the inside to give a concentration of 50 μ M. B. Control skin half. — short-circuit current ($\mu A/cm^2$) $\circ-\circ$ potential $\bullet-\bullet$ resistance ($k\Omega \cdot cm^2$).

the inside bathing solution of the frog skin results in an activation of the active Na^+ transport.

Effect on passive fluxes

To evaluate the effect of filipin on the permeability of the frog skin to substances, the transport of which can be accounted for by passive forces, transepithelial sucrose and chloride

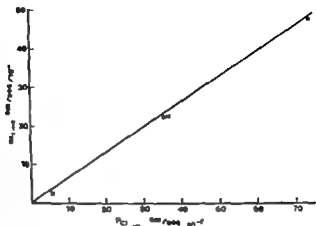


Fig. 1. The sodium surface flux against the chloride out flux. The estimates of sodium and chloride were measured simultaneously on the same skin after the addition of Filipin ($50 \mu\text{M}$) to its outside bathing medium.

is the formation of a pathway in the outward facing membrane that is insensitive to amiloride.

Fig. 5, which is representative for 8 expts., shows that the addition of Filipin to the inside bathing solution has no effect on the SCC when this was blocked by amiloride. After the outside bathing solutions were replaced by solutions without amiloride, the control skin did show the normal Filipin induced changes in the SCC. Thus the effect of Filipin added to its inside bathing solution is not due to a formation of an amiloride insensitive sodium pathway but is the result of an increase in the sodium flux via the amiloride sensitive sodium pathway.

Effect on cell volume

Although addition of Filipin to the inside had a very small effect on the transepithelial permeability it remained still an open question whether Filipin caused a large increase in the permeability of the forward facing cell membranes. If Filipin caused a large increase in the non-specific permeability of the forward facing cell membranes, the cells would swell. To

Table V. Effect of Amphotericin B on the permeability of the frog skin to chloride and urea. $50 \mu\text{M}$ amphotericin B in the inside bathing solution.

Ions	Number of expts	Control \pm S.E. A-B	Amphotericin B \pm S.E.	
			B-C	C-D
$\text{cm sec}^{-1} 10^{-4}$				
Lam Chloride	5	5.38 \pm 0.57	5.39 \pm 0.60	5.26 \pm 0.62
	6	5.00 \pm 0.61	4.57 \pm 0.75	4.93 \pm 1.58

In each expt. following 20 min equilibration for the isotopes the permeability across the skin was determined for each 1 h control period (period A-B). Amphotericin B was then added to the inside bathing medium. After the addition of the amphotericin B solution, the permeability was determined for 2 further 1 h periods (period B-C, C-D). All values are the means \pm S.E.

TABLE III Effect of filipin on the permeability of the frog skin to chloride and sodium. 50 μ M filipin the outside bathing medium.

Period	Number of expts.	Control \pm S.E. A B	Filipin \pm S.E.	
			B-C	C D
			cm sec ⁻¹ $\times 10^{-5}$	
Chloride outflux	8	2.76 \pm 0.55	28.1 \pm 4.9	43.1 \pm 5.3
Chloride influx	9	3.06 \pm 0.46	21.3 \pm 3.9	32.9 \pm 9.9
Sodium outflux	5	1.09 \pm 0.41	19.6 \pm 5.5	24.6 \pm 6.4

In each expt. following 20 min of eq. filtration for the isotope the permeability across the tissue is determined for one, 1 h period (period A-B). Filipin was then added to the outside bathing medium. After addition of the filipin solution, the permeability was determined for 2 further 1 h periods (periods C-D). All values are the means \pm S.E.

B to the inside bathing solution has no effect on the passive chloride and urea flux (Table 1). For both substances in- and outflux were measured. Thus the addition of amphotericin and filipin to the outside bathing solution resulted in a significant increase in the passive transepithelial fluxes, for the substances tested, whereas the addition of amphotericin and filipin to the inside bathing solution had no or very small effects on these fluxes.

Action of amiloride

Previous studies have shown that addition of amiloride to the outside bathing solution of the frog skin and the toad bladder inhibit the active transepithelial sodium transport (Sah and Smith 1970, Nielsen and Tomlinson 1970, Bentley 1968). This inhibition is believed to be caused by an interaction of amiloride with the specific sodium pathway (the sodium channels) in the outward facing membrane (Cuthbert and Shum 1974).

Fig. 4 shows an experiment in which the SCC was blocked by amiloride. The addition of filipin to the outside bathing solution caused full recovery of the SCC. In 10 expts. the addition of 50 μ M filipin to the outside bathing solution after the addition of amiloride resulted in 20-100% recovery of the SCC. Thus addition of filipin to the outside bathing solution

TABLE IV Effect of filipin on the permeability of the frog skin to chloride and sodium. 50 μ M filipin the inside bathing medium.

Period	Number of expts.	Control \pm S.E. A B	Filipin \pm S.E.	
			B-C	C D
			cm sec ⁻¹ $\times 10^{-5}$	
Chloride outflux	7	2.26 \pm 0.48	3.04 \pm 0.59	3.49 \pm 0.75
Chloride influx	7	1.84 \pm 0.34	2.38 \pm 0.43	2.51 \pm 0.44
Sodium outflux	5	1.19 \pm 0.22	1.66 \pm 0.16	1.71 \pm 0.66

In each expt. following 20 min of equalization for the isotope the permeability across the tissue is determined for one, 1 h period (period A-B). Filipin was then added to the inside bathing medium. After addition of the filipin solution, the permeability was determined for 2 further 1 h periods (periods C-D). All values are the means \pm S.E.

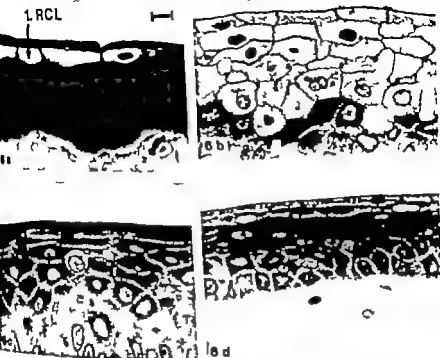


Fig. 6. Light micrographs of frog skin epithelium incubated with $50 \mu\text{M}$ filipin in the inside- or the outside bathing solution. 1 μm sections cleared with cedarstone blue. The bar on Fig. 6 corresponds to $10 \mu\text{m}$. (a) The skin incubated in 1 h with filipin in the outside bathing solution. (b) started swelling of the cells in the 1 RCL observed 6 h. The skin is incubated in 3 h with filipin in the outside bathing solution. (c) Swelling of the outermost 4-5 cell layers is observed, and the interspace system is closed by the swollen cells. (d) Control skin. 6 d. The skin is incubated in 3 h with filipin in the inside bathing solution. The cells in the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the skin in the 1 RCL see text.

to test the possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer's solution with OsO_4 to both chamber halves, to give a concentration of 1% OsO_4 in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding $50 \mu\text{M}$ filipin to the outside bathing solution. After 60 min incubation the volume of all the cells increases (Fig. 6 a) in the outermost layer of the *stratum granulosum* (the first reacting cell layer 1 RCL). After 3 h incubation the cells of the underlying 1-4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6 b). Salafino, Bentley and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swelto, Perriol and Lippe (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1 RCL. Even after 3 h of incubation with $50 \mu\text{M}$ filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6 d representative for 10 expts.). The effect of filipin (inside) on the cells in the 1 RCL was variable. In seven skins it had no effect, in two skins

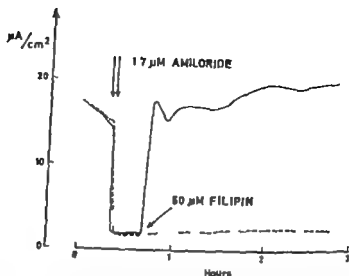


Fig. 4 Effect of filipin (outside) on the SCC of a frog skin where the SCC was blocked by amiloride. The arrows amiloride was added to the outside bathing solution of the skin halves to give a concentration of $17 \mu\text{M}$. At the other arrow filipin ($50 \mu\text{M}$) was added to the outside of the skin half represented by the solid line.

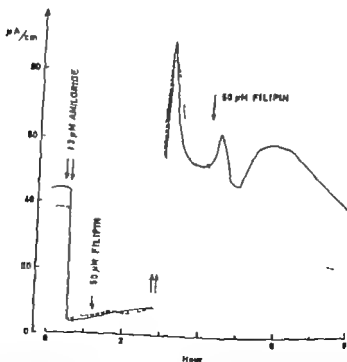


Fig. 5 Effect of filipin (inside) on the SCC of a frog skin where the SCC was blocked by amiloride. At the arrows amiloride was added to the outside bath solution of the skin halves to give a concentration of $1.3 \mu\text{M}$. At the dotted arrow $50 \mu\text{M}$ filipin was added to the inside bathing solution of the skin half represented by the dotted line. After 3 h of incubation the outside bathing solutions were replaced by fresh Ringer's solution and at the arrow (4 h) $50 \mu\text{M}$ filipin was added the inside bathing solution of the skin half represented by the solid line.

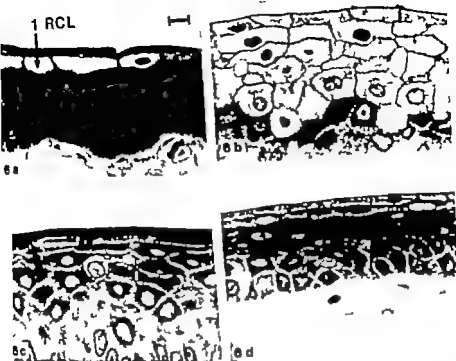


Fig. 6. Light micrographs of frog skin epithelium incubated with $30 \mu\text{M}$ filipin in the inside- or the outside bathing solution, 1 μm sections stained with toluidine blue. The bar on Fig. 6 corresponds to $10 \mu\text{m}$.

The skin incubated in 1 h with filipin in the outside bathing solution showed marked swelling of the cells in the 1 RCL (Fig. 6b). The skin is incubated in 3 h with filipin in the outside bathing solution. Marked swelling of the outermost 4-5 cell layers is observed, and the interspace system is closed by the swollen cells (Fig. 6c). Control skin (Fig. 6d). The skin is incubated in 3 h with filipin in the inside bathing solution. The cells in the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the skin in the 1 RCL see text.

test the possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer's solution with OsO₄ to both chamber halves, to give a concentration of 1% OsO₄ in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding $30 \mu\text{M}$ filipin to the outside bathing solution. After 10 min incubation the volume of all the cells increases (Fig. 6a) in the outermost layer of the stratum granulosum (the first reacting cell layer 1 RCL). After 3 h incubation the cells of the underlying 1-4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6b). Saladino, Bentsky and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swicko, Perrini and Lippe (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1 RCL. Even after 3 h of incubation with $30 \mu\text{M}$ filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6d representative for 10 expts.). The effect of filipin (inside) on the cells in the 1 RCL was variable. In seven skins it had no effect, in two skins

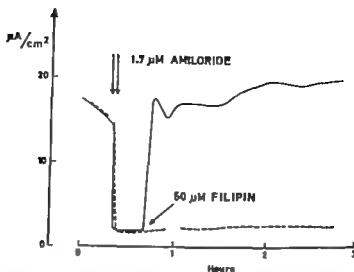


Fig. 4 Effect of filipin (outside) on the SCC of a frog skin where the SCC was blocked by amiloride. At the arrows amiloride was added to the outside bathing solution of the skin halves to give a concentration of $1.7 \mu M$. At the other arrow filipin ($50 \mu M$) was added to the outside of the skin half represented by the solid line.

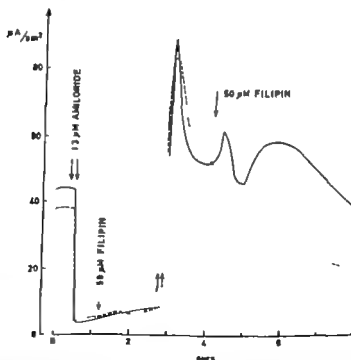


Fig. 5 Effect of filipin (inside) on the SCC of a frog skin where the SCC was blocked by amiloride. At the arrows amiloride was added to the outside bathing solutions of the skin halves to give a concentration of $1.3 \mu M$. At the dotted arrow $50 \mu M$ filipin was added to the inside bathing solution of the skin half represented by the dotted line. After 3 h of incubation the outside bathing solutions were replaced by fresh Ringer's solution and at the arrow (4 h) $50 \mu M$ filipin was added to the inside bathing solution of the skin half represented by the solid line.

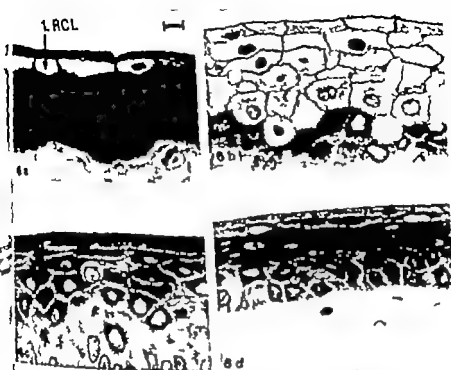


Fig. 6. Light micrographs of frog skin epithelium incubated with $50 \mu\text{M}$ filipin in the inside- or the outside bathing solution. 1, one section stained with toluidine blue. The bar on Fig. 6 corresponds to $10 \mu\text{m}$. (a) The skin incubated in 1 h with filipin in the outside bathing solution. started swelling of the cells in the 1 RCL is observed. (b) The skin is incubated in 3 h with filipin in the outside bathing solution. Swelling of the outermost 4-5 cell layers is observed, and the interspace system is closed by the swollen cells. (c) Control skin. (d) The skin is incubated in 3 h with filipin in the inside bathing solution. The cells in the lower cell layers did not show any tendency to swell. Concerning the effect of filipin on the skin in the 1 RCL, see text.

On this possibility expts. were performed in which the skins were incubated with filipin on the inside or on the outside from 10 min to 3 h. After the incubation, the skins were fixed by adding Ringer's solution with OsO_4 to both chamber halves, to give a concentration of 1% OsO_4 in the solutions bathing the skin. Fig. 6 (a, b) (representative for 7 expts.) shows the effect on cell volume of adding $50 \mu\text{M}$ filipin to the outside bathing solution. After 60 min incubation the volume of all the cells increases (Fig. 6 a) in the outermost layer of the *epithelium* (the first reaching cell layer 1 RCL). After 3 h incubation the cells of the underlying 1-4 layers are swollen to such an extent that the interspace system between them appears closed (Fig. 6 b). Salafino, Bentley and Trump (1969) have shown that the addition of amphotericin B to the mucosal side of the toad bladder causes a swelling of the cells in the epithelium, and Swello, Perriani and Lippe (1975) have shown the addition of amphotericin B to the outside of the frog skin results in a swelling of the cells in the 1 RCL. After 3 h of incubation with $50 \mu\text{M}$ filipin in the inside bathing solution the cells in the lower cell layers did not swell (Fig. 6 d representative for 10 expts.). The effect of filipin (Fander) on the cells in the 1 RCL was variable. In seven skins it had no effect, in two skins

it caused slight swelling—and in one skin a slight shrinkage. Hence addition of filipin to the outside results in an increase in the permeability of the outward facing cell membranes whereas the addition of filipin to the inside had no or a very small effect on the sodium permeability of the inward facing cell membranes.

Discussion

From the data presented above it appears that the addition of filipin to the outside bathing solution resulted in an increase in the passive transepithelial sodium, chloride, and sucrose fluxes and in a progressive swelling of the cells in the different cell layers. The addition of filipin to the inside bathing solution however had no effect on the passive transepithelial fluxes and no effect on the volume of the cells. The discussion will be separated in two parts. A, the effect of filipin on the transepithelial fluxes and B the effect of filipin on the progressive swelling of the cells in the different cell layers.

According to the two membrane hypothesis (Koefoed-Johnsen and Ussing 1958) the frog skin can be treated as composed of an "outward-facing membrane" which is selectively permeable to sodium ions but impermeable to potassium ions and permeable in a non-selective way to small anions like chloride. The "inward-facing membrane" is permeable to potassium and small anions, but impermeable to free sodium ions. The outward-facing membrane is located just beneath the cornified layer (Ussing and Windhager 1964 Farquhar and Palade 1966). The outward facing membrane consists of the outward-facing cell membranes of the outermost layer of the *s. granulosum* (1 RCL) kept together by tight seals. The inward-facing membrane is identical with the cell membranes limiting the extracellular space throughout the epithelial layer (Fig. 7 A). Thus a solute can permeate across the skin via two types of "pathways" via an extracellular route or via an intracellular route. A solute that permeates via the intracellular route has to cross both an inward and outward-facing cell membrane (Fig. 7 A). A component that only increases the sodium permeability of the outward-facing cell membranes should therefore have no effect on the passive sodium outflux (the inward-facing membranes is impermeable to free sodium) whereas a component which increases the sodium permeability of the inward-facing membrane should increase the passive sodium outflux.

Addition of filipin to the outside results in an increase in the amiloride insensitive sodium transport (Fig. 4), thus filipin increases the sodium permeability of the outward facing cell membrane. Furthermore it has been shown by Nielsen (1971 1972) that filipin and anophotericin B increase the potassium permeability of the outward facing cell membrane, too. An increase in the sodium and potassium permeability of the outward facing membrane would result in an increase in the cellular sodium concentration and a decrease in the cellular potassium concentration. These changes in the cellular compositions should result in an increased sodium transport. Such an increase was only observed in half the experiments. But if the addition of filipin to the outside bathing solution makes the outward facing membrane so permeable to sodium that the sodium flux into the cells exceeds the capacity of the sodium pump, the cells would undergo colloid-osmotic swelling and eventually burst. The

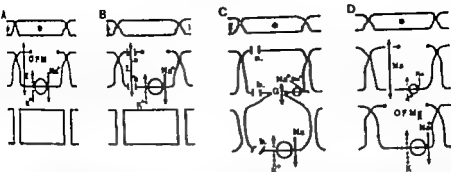


Fig. 7 Diagram of the 3 outermost layers of the isolated frog skin. The top layer (the hatched layer) is the corneum. The underlying layer is the *stratum granulosum* (1 RCL). The 1 RCL (1 reacting cell layer) is electrically permeable to sodium (indicated by the dots). 7 A. OFM, outward facing membrane. I, intracellular space. 7 B. and C, disintegration of the outward (a) and (b) the inward facing cell membranes. 7 C. Cl⁻ ion channel formed as a result of the filipin induced swelling of the 1 RCL. For further information see text. 7 D. Filipin induced changes in the 1 RCL could result in formation of new outward facing cell membranes (OFM₂) in the underlying cell layers. For further information see text.

variable effect of filipin on the SCC could readily be understood if filipin in some skins results in a fast and extensive cytolysis of the cells. The data of Fig. 6 A show that addition of filipin to the outside solution results in a swelling of the cells.

The swelling of the cells would most likely result in a partial disintegration of both the inward and the outward facing cell membranes (Fig. 7 B). A partial disintegration of the inward and outward facing cell membranes would result in a nonspecific increase in the passive transepithelial permeability which could explain the increase in the passive transport of sucrose, chloride and sodium (Tables II, III and Fig. 3).

Since the addition of filipin to the inside bathing solution has no effect on the sodium effect, filipin (according to the two membrane hypothesis explained above) has no effect on the sodium permeability of the inward facing cell membrane, and therefore it should have no effect on the cell volume and the passive transepithelial permeability. That this interpretation is correct appears from the data of Tables II, IV and Fig. 6 D. The addition of filipin to the inside results, however, in an increase in the active sodium transport (Fig. 2, Table II). This is probably caused by an indirect effect of filipin on the potassium permeability of the inward facing membrane, mediated by calcium entry (Nielsen 1976).

Many studies show that the presence of sterols in the membrane is a requirement for polyanionic antibiotic sensitivity (for references see Norman *et al.* 1972). If as suggested by Kruijff and Demel (1974) the filipin induced permeability changes are a result of filipin forming large filipin-cholesterol complexes in the membrane, the outward-facing membrane must be assumed to have a high cholesterol content, because filipin *per se* only increases the non-specific permeability of the outward-facing membrane; the cholesterol content of the inward-facing membrane must be low.

After 1 h of incubation with filipin (outside) the cells of the 1 RCL are swollen and have a secretory appearance (Fig. 6 A) none the less most of the skins are still able to generate a high SCC, whereas the skins are unable to generate a SCC when nearly all the cells in the

epithelium are swollen. Therefore, presumably after 1 h of incubation the 1 RCL consists of cells which are unable to produce a SCC, and the cells in the underlying cell-layers generate the SCC. Under these circumstances sodium can diffuse easily from or through the cells in the 1 RCL to the cells in the underlying layers. However Voûte and Ussing (1968) have shown that only the 1 RCL swells during short-circuiting. This result, and the kinetic analysis of sodium and lithium movements in the isolated frog skin by Morel and Leblanc (1973) show that the diffusion from cells in one layer to those in the adjacent ones is very slow. This implies that in normal frog skin the cells of the 1 RCL are responsible for the major part of the active transepithelial sodium transport. That is supported by expts. of Voûte and Ussing (1970) which show that expansion of the interspace system have no effect on the SCC (in these expts. the interspaces (apart from the tight seals) were so expanded that the cells only were in contact via the desmosomes). Since filipin *per se* only increases the non specific permeability of the outward-facing membrane of the 1 RCL, this effect cannot explain the increase in the direct sodium flow from cell to cell in neighbouring layers. The following two models (A and B) can explain the increase in the direct passing of sodium from one cell layer to the next. A. when the cells in the 1 RCL swell they come in contact with the cells of the underlying cell layer resulting in the formation of new gap junctions (Fig. 7 C) or B, the filipin-induced changes in the 1 RCL could result in the release of a signal which caused a change in the outward-facing membrane of the underlying cell layer so that this cell layer becomes sensitive to filipin (Fig. 7 D (e.g. these cells might form a new outward-facing sodium selective membrane). This hypothesis can explain how an epithelium with all the cells in the 1 RCL having a marked necrotic appearance still can transport sodium and it can explain the observed progressive swelling of the underlying cell layers.

Experiments are in progress to investigate whether the filipin induced necrosis of a cell layer results in the formation of gap junctions or results in the formation of a new outward facing cell membrane of the cells facing the necrotic cells.

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Loewenstein (1973) has shown that junctional passageways (gap junctions) form rapidly where cells

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Extracellular Potassium Concentration in Juvenile and Adult Rat Brain Cortex during Anoxia

By

ANKER JON HANSEN

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Abstract

HANSEN A. J. Extracellular potassium concentration in juvenile and adult rat brain cortex during anoxia. *Acta physiol. scand.* 1977 99 412-420

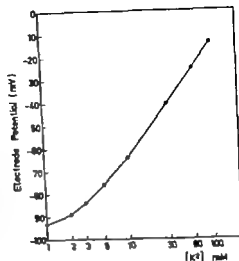
The extracellular K^+ concentration, ($[K]_o$), in the brain cortex of rats at different ages was measured by means of K^+ -sensitive microelectrodes. $[K]_o$ was between 3 and 5 mM at all ages. Following nitrogen asphyxiation there was an increase in $[K]_o$ which exhibited a sigmoid pattern in every age group. Firstly there was a slow rate of rise which was followed by a sudden, steep increase where the $[K]_o$ rose in about 70 mV. During the subsequent 5-10 min a plateau value of about 90 mM was reached. The prominent difference between the age groups was the time until the steep rise began. The time to the steep increase in $[K]_o$ and the $[K]_o$ at the start of the steep increase was inversely related to the age of the animal. A close relation was found between the time to the start of the steep increase and the time to the last gasp during anoxic breathing ($r=0.98$). It is suggested that the different resistance to anoxia in young and adult animals is related to differences in the ability to keep near normal potassium gradients across the cells in the brain.

It is well established that young animals survive longer periods of anoxia than adults. It has been shown that the rate of rise in the potassium concentration of the cerebrospinal fluid (CSF) following brain ischemia in rats was correlated with the age of the animal (Hansen 1976b). Since a high concentration of potassium extracellularly seriously impairs brain function by depolarizing the nerve cells it was thought that the ability to keep extracellular potassium concentration ($[K]_o$) near normal values would reflect the tolerance of the brain to a period of anoxia. Due to the long diffusion distance from the tissue to CSF the CSF potassium values are distorted in relation to tissue values in the unsteady state. In order to circumvent this problem K^+ selective microelectrodes were used to measure $[K]_o$ directly in the brain cortex. The aim of the investigation was therefore to examine whether differences in the ability to regulate $[K]_o$ during anoxia in the brain cortex of immature and mature rats might be correlated to the known differences in tolerance of anoxia. A preliminary report has been given (Hansen 1976a).

Methods

Sprague-Dawley rats were studied. Their ages and numbers were as follows: 4 days, 4 animals; 7 days, 4 animals; 12 days, 5 animals; 16 days, 6 animals; 24 days, 4 animals and adults, 7 animals. The rats were

Fig. 1 Calibration curve of double-barrelled K⁺ microelectrodes obtained in solutions of known concentrations of KCl with NaCl added to ensure constant ionic strength



maintained with pentobarbital administered I.p. (20–30 mg/kg in young rats and 50 mg/kg in the adults), tracheotomized and placed on a heated table. The body temperature was maintained at 37°C by a servo-controlled heating system. The rats breathed spontaneously and the respiration rate was measured with a small flowmeter inserted in the tracheal tube and the ECG was recorded from needles in the front legs. Blood pressure was monitored only in the adults, via a catheter in the femoral artery. The head of the animal was secured in a headholder. Since the heads of the juvenile rats are not sufficiently ossified to withstand the pressure from the cranium, their heads were immobilized between two pointed forks. Thereafter a trephine opening 3–4 mm in diameter was made in the left parietal bone. In the adults the dura was peeled with sharp needles, but in the other animals it was left intact. The respiratory rate, ECG and the blood pressure were recorded on a 4-channel Beckman raster. The animals were made anoxic by letting them breathe pure nitrogen.

Fluorine microelectrodes

The electrodes were made as described by Zeebén *et al.* (1974). Double-barrelled micropipettes were pulled from two aligned capillary tubes, each were offset 1 cm along the long axis. This produced pipettes with one long and one short barrel. The tip diameter was about 1 µm as determined by light microscopy. The long barrel was silicized by exposing the open backside to the vapor of dichlorodimethylsilane for 1 h. The pipettes were then dried in an oven at 100°C for 1 h. The shank of the silicized barrel was filled with potassium exchange resin (Cormag 477317) using a thin capillary tube. Usually the exchanger would fill the tip immediately. The rest of the long barrel and the short barrel was then filled with 150 mM KCl. Air bubbles when the pipettes were removed using the principle of Zeebén (1971). A 50 µm heated wire was applied to the outside of the electrode at the air-water or air-ion exchanger interface furthest from the tip. The water or exchanger were thereby distilled towards the tip, while the bubble moved away from the tip. Since the exchanger has a higher boiling temperature than 150 mM KCl solution, bubbles in the exchanger were removed before filling the reference barrel.

The potential arising between the barrels is a measure of the K⁺ activity at the tip. The electrodes were calibrated in solutions containing different concentrations of KCl with NaCl added to ensure constant ionic strength. The calibration curve derived in this way was used to convert the measured K⁺ activity to K⁺ concentration (Fig. 1). The potential of the reference barrel varied in most electrodes less than 3 mV in different test solutions. Electrodes with higher values were discarded. The electrodes typically showed a 40–60 mV change for a 10-fold change in K⁺ concentration. The resistance of the potassium barrel varied between 10⁷ and 10⁸ Ω. Ag/AgCl connections led from both barrels via shielded cables to a Keithley 616A electrometer 604 (input impedance 10¹⁴ Ω). The potential between the reference barrel and the ground was measured by recording the negative gain output with respect to a grounded glass tube filled with 150 mM KCl solidified in agar. The K⁺ activity and the tissue potential were recorded as individual

TABLE 1 Extracellular K^+ concentration in brain cortex of rats at different ages. The data, expressed in mM, are means \pm S.D.

	Age (days)					
	4	7	12	16	24	Adult
$[K^+]_e$	4.6 ± 1.3	2.9 ± 0.4	3.4 ± 0.6	3.1 ± 0.5	4.3 ± 0.4	1.0 ± 0.5

channels on a pen writer. The response time of the system was less than 1/2 s. The animal was grounded in the agar filled glass tube buried in a neck muscle.

The brain electrode was inserted with a micro-manipulator controlled by a step motor. In all animals the K^+ activity was measured at a depth of 0.5 mm. The K^+ activity recorded at this depth was constant to at least 5 min before nitrogen was administered to the animal. The electrodes were calibrated before and just after the experiments. If there were significant differences (> 2 mV) between the two calibrations the results were discarded.

Results

$[K^+]_e$ resting level

The base-line value of $[K^+]_e$ in the brain cortex of rats at different ages are shown in Table 1. At all ages the resting values were between 2.9–4.6 mM.

$[K^+]_e$ in the brain following anoxia

The rise following nitrogen inhalation exhibited the same pattern at all ages (Fig. 2). Firstly there was a slow rate of rise, which was followed by a sudden steep increase, where the $[K^+]_e$ rose to about 70 mM. Hereafter it slowly increased until it reached a final level of about 90 mM after 10 min. The prominent difference in the response between the age groups was the time until the steep increase began. In the 4-day-old rat it took about 20 min to reach the steep increase, while in the adult it took only 1.5 min. In fact, the time delay was inversely related to the age of the animal (Fig. 3). In the 4, 7 and 12-day-old rat the steep increase began at a $[K^+]_e$ of about 25 mM but in rats older than 12 days it began at about 10 mM.

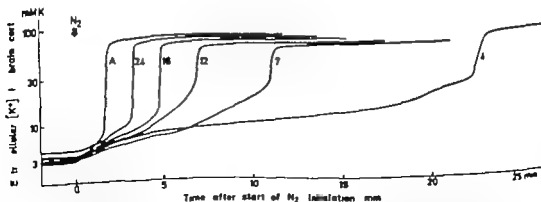


Fig. 2. $[K^+]_e$ in the brain cortex of rats at different ages following exposure to nitrogen. The number indicates the age (days) of the animal. A stands for adult. The figure shows representative experiments.

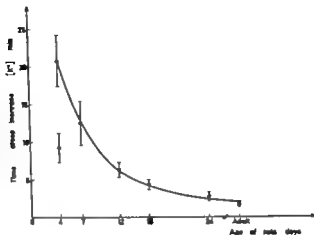


Fig. 3 The time to the start of the steep increase in $[K^+]_b$ in the brain after following nitrogen inhalation in rats at different ages (\circ) and following removal of the heart in 4-day-old rats (\circ). 1 S.D. is indicated by vertical bars.

Fig. 4) The slope of the steepest part of the K^+ curve was related to the age of the animal (Fig. 5).

The steep increase in $[K^+]_b$ was in all ages accompanied by a negative shift in the tissue potential, called anoxic depression (Leilo 1947; Bureš and Burešová 1957).

$[K^+]_b$ and last gasp following anoxia

To investigate whether there was a relationship between the time to the last breath following anoxia and the time in the steep increase in $[K^+]_b$, a plot was made and the correlation coefficient was calculated (Fig. 6). There was a close relationship between the two parameters ($r = 0.98$).

$[K^+]_b$ changes following brain ischaemia

To examine the significance of the circulation on the $[K^+]_b$ in the brain during anoxia, the heart was removed in four 4-day-old rats. Within 2 min the rat was mounted in the head-

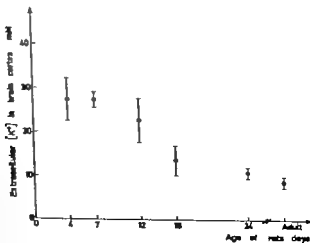


Fig. 4 $[K^+]_b$ at the start of the steep increase in $[K^+]_b$ in brain cortex of rats at different ages following nitrogen inhalation. 1 S.D. indicated by vertical bars.

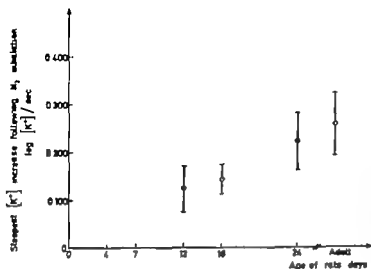


Fig. 5. The slope of the steep increase in $[K]_i$ in the brain cortex of rats at different ages following nitrogen inhalation. S.D. is indicated by critical bars when exceeding the symbols.

holder and the electrode inserted in the brain cortex. The $[K]_i$ increased faster in these animals and the steep increase was reached in 9–10 min instead of 20 min in similar rats with intact cardiovascular system (Fig. 3) but the $[K]$ at the beginning of the steep increase was not changed. In 4 adults the heart was arrested by injection of concentrated $MgCl_2$ through the femoral vein. Previous expts with Evans blue added to the $MgCl_2$ solution showed no visible staining of the brain during this procedure. This method of arresting the circulation did not affect the course of events significantly.

Reversibility of the anoxic $[K]_i$ increase

In other expts, the nitrogen supply was disconnected in adult rats just after the steep increase in $[K]_i$ had occurred whereafter the animals were allowed to breathe air. In most of the

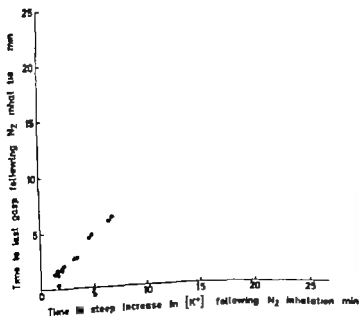
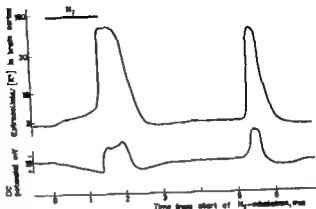


Fig. 6. The correlation between the time to the last gasp and the time to the start of the steep increase in $[K]_i$ in brain cortex of juvenile and adult rats $r = 0.975$.

Fig. 7. Changes in $[K]_o$ and the extracellular potential in the brain cortex of an adult rat during and after nitrogen substitution. Note: 1. synchronous, transient increase in $[K]_o$ and the change in a most potential characteristic 'late spreading depression'.



and $[K]_o$ in the brain returned to preanoxic levels (Fig. 7). This procedure was sometimes slowed by an increase in $[K]_o$ characteristic of Lells spreading depression (Vyskočil *et al.* 1972). A prerequisite for the return of the $[K]_o$ was that the cardiovascular system responded to the air breathing by an increase in blood pressure. Otherwise the steep increase was irreversible.

Discussion

$[K]_o$ level following anoxia. It was shown in the present investigation that $[K]_o$ in the brain cortex was 3–5 mM in juvenile and adult rats. This is in accordance with the results obtained by Mizuki *et al.* (1974) who studied immature rabbits.

Following nitrogen substitution a slow rise in $[K]_o$ was found which was later followed by a steep increase reaching a final $[K]_o$ level of about 90 mM (Fig. 2). The fact that $[K]_o$ rose in a sigmoid fashion following anoxia has no simple explanation. The cells in the brain are able to lose their K⁺ following anoxia since the high intracellular K⁺ concentration is maintained by energy dependent processes. The virtual impermeability of the blood-brain barrier to K⁺ (Croce and Thompson 1970, Hansen and Lund-Andersen 1976) prevents a rapid escape of the K⁺ to the blood and thus an increased $[K]_o$ is maintained.

The initial increase in $[K]_o$ occurred at a relatively slow rate and may be caused by a passive leak of K⁺ not counteracted stoichiometrically by the ion pumps in the cell membrane, due to a reduced supply of ATP. A reduction in the extracellular space would also increase the $[K]_o$, but the changes observed from conductivity measurements (Van Harrewald 1977) are too small to account for the observed changes. The duration of the initial rise in $[K]_o$ became steadily shorter during ontogenesis (Fig. 2). This might be explained by a faster depletion of ATP in the brain cortex due to the increase in cerebral metabolic rate with age (Duffy *et al.* 1975) in combination with a decrease in the ability of the heart to withstand anoxia (Davies *et al.* 1959).

Benčlová *et al.* (1957) studied in isolated heads of adult rats the time to the negative shift in tissue potential, which is a phenomenon that accompanies the steep increase in $[K]_o$.

They found that the time was prolonged by decreasing the cerebral metabolic rate with barbiturates and hypothermia. Glucose administered *ip* increases the glucose stores in the brain cortex (Ljunggren *et al* 1974). Anaerobic glycolysis might hereby provide ATP for a longer time during anoxia. Hansen (unpublished) found that pretreatment with glucose in the adult rat delays the occurrence of the steep increase in $[K]_o$ following brain ischemia. Thus, the duration of the initial increase in $[K]_o$ seems to be dependent on the ability of the brain to maintain sufficient ATP levels, and the transition to the steep increase may be related to a low ATP level in the brain, but no experimental data are available. At the time of the negative shift in tissue potential there is a decrease in the extracellular space of the brain cortex (Van Harreveld 1972) due to cellular uptake of Na, Cl and water but since the decrease in space computed from conductivity measurements only amounts to about 50% it cannot solely explain the magnitude of the steep rise in $[K]_o$. Another possibility is an increase in the K^+ permeability (P_K) of the cell membrane. The change in P_K could be induced by an increase in $[K]_o$ since this would lead to a depolarization of the cells and thereby to an increase in the P_K of the neurons (Hodgkin and Huxley 1952). It is well known that application of KCl on the cerebral cortex elicits a spreading depression (Loeb 1944), which involves a rapid rise in $[K]_o$ and a concomitant negative shift in the tissue potential similar to the events during anoxia (Fig. 7). However, in rats younger than 12 days the application of KCl did not elicit a spreading depression, but only a local, negative potential shift (Dex and Eldelberg 1967). The present study has shown that a negative shift in tissue potential at all ages accompanies a rapid rise in $[K]_o$. Lothmann *et al* (1975) found that if the $[K]_o$ exceeded 12 mM following electrical stimulation a spreading depression invariably followed. Assuming that the mechanism is the same for the steep increase in $[K]_o$ during a spreading depression and following anoxia, these results suggest that the initial increase in $[K]_o$ during anoxia is responsible for eliciting the subsequent increase in $[K]_o$.

Also a release of Ca^{++} from the mitochondria due to a reduced level of ATP (Kamenitz 1975) and cellular swelling (Hendil and Hoffmann 1974) which accompanies anoxia, could induce a rise in P_K . Cellular swelling is not a likely possibility since shrinkage of the cells in the brain with a hypertonic solution before anoxia did not affect the time to the steep increase in $[K]_o$ following anoxia (Hansen unpublished). However, increase in intracellular Ca^{++} might be a possible candidate for eliciting the assumed increase in P_K following anoxia, but data on this matter are not available.

From Fig. 2 it appears that the $[K]_o$ at the beginning of the steep rise in $[K]_o$ decreases with age. The reason for this is unclear, but a possibility might be that the immature neuron is less permeable to K^+ than that of the adult. A sufficient depolarization for a rise in P_K could thus only be reached with a higher $[K]_o$. Supporting this idea is the study of Mutani *et al* (1974) who found that immature rabbit brain cortex could generate epileptiform activity with a $[K]_o$ of at least 20 mM.

The rate of rise of $[K]_o$ during the steep increase became steadily faster during ontogenesis (Fig. 5). An increase in surface-volume ratio of the neurons (Eayrs and Goodhead 1959) and a decrease in the extracellular space (Vernadakis and Woodbury 1962) could explain this finding.

In the discussion I have focused upon the neurons as the source of K^+ during the steep

known in $[K]_e$. However it is not known if this is correct or if the glia are involved. In the future we shall try to investigate this problem.

$[K]_e$ level and anoxic survival. The gradual decrease in the tolerance to anoxia which occurs during ontogenesis (Favckes *et al.* 1941) may be related to the ability to maintain the normal $[K]_e$ in the brain under anoxic stress. Several studies corroborate such an idea. Adolph (1959) found survival times for young and adult rats exposed to nitrogen similar to the time intervals for the appearance of the steep increase in $[K]_e$ reported in this study. Duffy *et al.* (1975) found that 7-day-old rats survived nine times longer than adults, while the study has shown that the time to reach the steep increase in $[K]_e$ in 7-day-old rat was eight times longer than in the adult. The criteria for survival in the above mentioned studies was the ability of the animals to recover spontaneously after anoxia. Other studies have shown that the survival after anoxia parallels persistent gasp reflex and have therefore used the duration of gasping during anoxia as the length of anoxic survival (Selle and Witten 1941). In the present investigation a correlation coefficient of 0.98 was found between the time to the last gasp and the time to the steep increase in $[K]_e$.

The steep increase in $[K]_e$ following anoxia is a reversible phenomenon if the cardiovascular system is still functioning. A close relationship has been found between cardiac content of carbohydrates and the survival of rats at different age following anoxia (Stafford and Weherall 1960). In this study the time until the steep increase in 4-day-old rats was delayed by stopping the heart in comparison with animals inhaling nitrogen, while no difference was observed in the adult. It should therefore be emphasized that the reported change in the ability to regulate $[K]_e$ of the brain cortex following anoxia which takes place during ontogenesis is due to different tolerance of both the brain and the cardiovascular system to anoxia. The evidence summarized above supports the idea that the different resistance to anoxia in young and adult animals is related to the ability to keep near normal K⁺ gradients across the cells in the brain.

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Distribution of Coronary Blood Flow in the Left Ventricular Wall of Dogs Evaluated by the Uptake of Xe-133

By

HENNING BAGGER

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Abstract

*H Distribution of coronary blood flow in the left ventricular wall of dogs evaluated
by uptake of Xe-133 Acta physiol. scand. 1977 99 421-431*

Uptake of coronary blood flow was estimated in anesthetized dogs by counting the activity in
the left ventricular free wall immediately after bolus injection of Xe-133 into the aortic
infundibulum. The uptake of isotope was observed between the apex and the base of the heart,
and supplied by the anterior descending and circumflex branches of the left coronary artery. In
the endo- and epicardial halves of the wall. In most experiments bolus injection of the isotope
into the coronary artery was followed by a difference in activity between areas supplied by the left
anterior descending and left circumflex branches. This indicated inadequate mixing of blood and isotope
at some of the artery. The uneven distribution did not result in differences between the endo- and
epicardial concentrations. The results from one normal, anesthetized dog in which tissue activities
were measured after constant rate infusion of Xe-133 into the left coronary artery for 8 min were in ac-
cord with the general assumption of equal endo- and epicardial volumes of distribution (values of Z).

estimation of local blood flow from the rate of disappearance of intramyocardial deposits
of ^{133}Xe (Kirk and Honig 1964) and ^{133}Xe (Harley, Harper and Estes Jr 1966, Brandt,
and McGregor 1968) have shown a higher tissue flow in the endo- than the epicardial
half of the left ventricular wall of anesthetized dogs. Myocardial uptake of continuously
infused ^{133}Xe (Love and Burch 1957, Mohr and DeBra 1967, Mathes and Rival 1971) and
thymidine (Griggs and Nakamura 1968) have indicated a flow (vol time⁻¹ weight⁻¹)
in the endocardial half of the ventricular wall equal to or higher than that of the epicardial

In previous papers (Andersen, Bagger and Gatzsche 1969, Bagger 1972) the two
flows were estimated employing the same isotope. Wash-out of ^{133}Xe after local in-
jection into the left ventricular wall showed higher endo- than epicardial flow while myocar-
dial activity immediately after injection of xenon into the left ventricular cavity was higher
endo- than the epicardial tissue blocks. This implied that conflicting results may

arise from differences in the way of administration of a particular tracer. Furthermore the results (Bagger 1972) indicated that, when injected into the left ventricular cavity Xe-133 is able to cross the endocardial surface like Rb-86 (Moir and DeBra 1965; Myers and Hon 1966).

The distribution of radioactive microspheres with a diameter larger than 15 μm tend to exaggerate flow differences in the left ventricular wall of dog hearts (Yipintsoi *et al.* 1973). However, smaller microspheres (7–15 μm) injected into the left atrium of normal anesthetized dogs in some reports show 10–40% higher endo- than epicardial flow (Fortum *et al.* 1971; Flarøng *et al.* 1975; Walter *et al.* 1975) while in others reveal a uniform flow (Becke, Fortuin and Pitt 1971; Buckberg *et al.* 1972 and 1975; Fixler, Saunders and Suggs 1974).

In the previous report (Bagger 1972) adequate mixing of blood and tracer was obtained after bolus injection of Xe-133 into the left ventricular cavity, but the use of tissue activity as an indicator of regional blood flow in the left ventricular wall was disturbed by the uptake of isotope across the endocardial surface. When Xe-133 is injected into the aortic root or into the left coronary artery, transendocardial uptake of isotope is avoided. If adequate mixing of xenon and blood can be obtained by these routes of injection the epi- and endocardial distribution of activity might be an indicator of flow distribution. The present study was performed in order to see if adequate mixing of isotope and blood is obtained after bolus injection of Xe-133 into the aortic root and into the left coronary artery. Furthermore the epi- and endocardial distribution of activity was measured in order to discuss its relation to flow to these regions. Xe-133 was used in the present experiments for direct comparison of the results to those previously obtained (Bagger 1972).

The fractional volumes of distribution for xenon and other diffusible tracers used for myocardial blood flow measurements are generally assumed to be identical in the epi- and endocardial part of the ventricular wall (e.g. Brandt, Farn and McGregor 1968). This assumption was controlled in one normal anesthetized dog in which tissue activities were measured after constant rate infusion of the isotope into the left coronary artery for 8 min.

Methods

15 mongrel dogs of both sexes (19–40 kg) were used for the experiments. In 7 dogs (group 1) the uptake of Xe-133 was measured after injection of the isotope into the aortic root. In 7 other dogs (group 2) and also in the dog used for constant rate infusion, xenon was injected into the left coronary artery.

Four dogs in each of the two groups were premedicated with a subcutaneous injection of methadone 20 mg, tropine 0.5 mg and 2 propyl-10-(dimethylamino-propyl)-flutazam (Combelene®) 0.5 mg. 30 min later they were anesthetized with thiomebumal sodium (Leopental®) 8 mg/kg. Additional doses of the barbiturate (50–100 mg) were given as required. The other dogs were anesthetized with thiomebumal sodium (Leopental®) or pentobarbital (Nembutal®) 25 mg/kg and similarly supplied with additional 50–100 mg doses. Premedication was used in order to reduce the dose of barbiturate, thereby diminishing the negative inotropic effect on the heart. As the result from pre- and non-premedicated dogs were not different, they are not kept separated in this report.

The dogs were ventilated with atmospheric air through an endotracheal tube by means of an Engstrom ventilator. Additional oxygen was supplied to two dogs in group 2 in which there was a low P_{O_2} but normal P_{CO_2} after thoracotomy. This was probably due to atelectases which might develop after opening of the thorax.

A Dacor® left coronary artery catheter was introduced through the right femoral artery and the tip placed temporarily in the descending aorta. A polyethylene catheter was introduced through the left femoral

TABLE I. Hemodynamic and blood gas parameters of the 14 dogs, measured just before injection of Xe-133. The figures are mean values \pm S.D. N = number of dogs.

	Aortic pressure, mmHg		Heart rate beats min ⁻¹	P O ₂ mmHg	P CO ₂ mmHg	pH
	systolic	diastolic				
no vent. occlusion	133 \pm 27 N=7	102 \pm 25 N=7	144 \pm 45 N=7	71 \pm 16 N=7	35 \pm 5 N=7	7.47 \pm 0.08 N=7
ventr. artery ligation	128 \pm 12 N=7	107 \pm 13 N=7	154 \pm 13 N=7	73 \pm 14 N=5	31 \pm 6 N=7	7.40 \pm 0.05 N=7

ly and the tip placed in the descending aorta to allow continuous observation of pressure (together with simultaneous recording of the ECG). Thoracotomy was performed on the left side and the pericardium was retracted in the dogs of group 1. The tip of the Dacor catheter was positioned just above the aortic arch, just passing at the region of the left coronary artery in group 2 and in the dog exposed to continuous aortic occlusion, the tip of the catheter was placed just within the left coronary artery. In all experiments a part of the catheter was combined under fluoroscopy by injection of radiopaque material. 20-30 minutes after the injection the oxygen and carbon dioxide tensions and the pH of arterial blood were measured. 435 μ mol of saline solution was then injected as bolus through the Dacor catheter and the heart immediately beat.

13.41 mCi and 0.85-0.14 mCi Xe-133 were injected in group 1 and 2 respectively. During the count period 0.86 mCi of ²²NaCl solution was injected per min for 6.4 min, during which period the activity was detected normally by scintillation detector and recorder.

After removal the left ventricular cavity was flushed with water through the left stream. This was to remove the blood from the endocardial surface. The heart was then frozen in a mixture of dry ice and acetone (-75°C). The apex was removed and the heart was sliced at right angles to the long axis. In each slice the left ventricular free wall was divided into blocks. Each block was further subdivided into epi- and endocardial halves and placed in previously weighed, cooled, closed tubes. In the experiment of constant infusion of xenon, the blocks of one slice were divided separately as described above, while the blocks of the two other slices were divided as epi- and endocardial halves after removal of the epicardium with the major vessels. The activity was counted in a well-type scintillation detector with window tuned around the 81 keV peak and expressed as cps/100 mg of tissue.

Results

The hemodynamic parameters, blood gas values and pH just before injection of xenon, are shown in Table I. The mean values of the two groups are not significantly different. In the group with left coronary artery ligation the arterial oxygen tensions of the two dogs supplied with additional oxygen were 164 and 188 mmHg. These figures are not included in the values of the table.

The time interval between bolus injection and heart excision was 14.3 \pm 2.5 (S.D.) and 5.3 \pm 6.6 (S.D.) in group 1 and 2 respectively, the longest interval being 26 s. The interval between excision and freezing of the heart was 2.42-3.07 min, measured in 3 dogs of group 1. The interval between freezing and placing of the last tissue in its tube was less than 120 min.

A representative result of the distribution of Xe-133 in the epi- and endocardial halves of the left ventricular wall after injection of the isotope into the aortic root is shown in Fig. 1. The activity concentrations (cps/100 mg of tissue) of the tissue blocks of each slice are plotted in the direction from the left anterior descending branch towards the circumflex

arise from differences in the way of administration of a particular tracer. Furthermore the results (Bagger 1972) indicated that, when injected into the left ventricular cavity Xe 133 is able to cross the endocardial surface like Rb-86 (Moir and DeBra 1965, Myers and Hon 1966).

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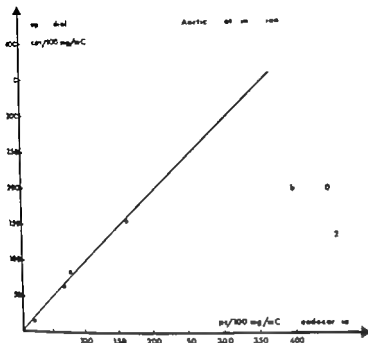
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A Dacor® left coronary artery catheter was introduced through the right femoral artery and the tip placed temporarily in the descending aorta. A polyethylene catheter was introduced through the left femoral



Comparison of mean epi- and endocardial activity (cpa/100 mg/hrC injected) of the left ventricular 17 days after aortic bolus injection.

is not different from unity ($p = 0.50$), the correlation coefficient 0.98 and the intercept 5.6 ± 19.5 (S.D.). The mean ratio of epi- to endocardial activity concentrations 1.00 ± 0.03 (S.E.). Again no correlation was found between the ratio of epi- to endocardial activity concentrations and the time taken to excise the heart.

constant infusion of Xe 133 into the left coronary artery of one dog a slight increase in activity recorded by external counting was still observed during the last part of the infusion period. In this dog the ratio of epi- to endocardial activity concentrations for all blocks of the left ventricular free wall was 1.02 ± 0.16 (S.D.), $n = 34$. The slice with epicardium showed a ratio of 1.06 ± 0.13 (S.D.), $n = 13$ while the ratio for the two slices without epicardium was 1.00 ± 0.18 (S.D.), $n = 21$. These mean values are not significantly different ($p = 0.30$).

Discussion

In the group of dogs subjected to aortic root injection no differences were observed in concentrations of xenon between the apex and the base of the heart or between areas supplied by the left anterior descending and circumflex branches of the left coronary artery. These data are in accord with those obtained after injection of Xe-133 into the left ventricular cavity (Bagger 1972) and thus indicate adequate mixing of isotope and blood before the left coronary artery branches. They do not confirm the autoradiographic picture of flow variations

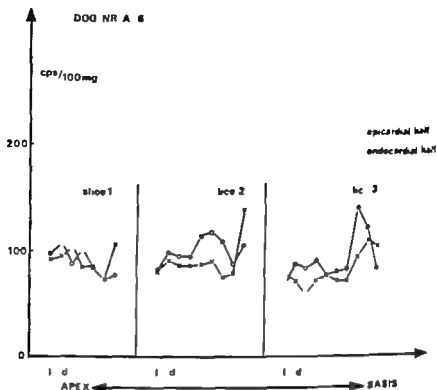


Fig. 1 A representative result of the distribution of Xe-133 in the epi- and endocardial halves of the slices of the left ventricular wall, after bolus injection of the isotope into the aortic root. The activity of the blocks of each slice is shown the direction from the left anterior descending towards the circumflex branch of the left coronary artery

supplied area. The slices are numbered in the direction from the apical region towards the base of the heart. The level of activity is constant from slice to slice, and constant in the tissue blocks of each slice independent of blood supply from the anterior descending or circumflex branch of the left coronary artery. Fig. 2 shows a comparison between the mean epi- and endocardial activity concentrations (per mCi injected) of all seven dogs exposed to aortic root injection. Each point represents a mean value of about 25 tissue blocks from one heart. The slope of the regression line (b_1) is 0.96 ± 0.05 (S.D.) (which is not different from unity $p > 0.40$), the correlation coefficient 0.99 and the intercept (y_0) 1.2 ± 9.5 (S.D.). The mean ratio of epi- to endocardial activity concentrations was 0.98 ± 0.05 (S.E.). No correlation was found by comparing the ratio of epi- to endocardial activity concentrations of each experiment with the time taken to excise the heart.

Fig. 3 and Fig. 4 show results obtained after injection of Xe-133 into the left coronary artery in two dogs. Fig. 3 shows an almost equal distribution of activity concentration in the regions supplied by the left anterior descending and circumflex branches, while in Fig. 4 there is considerably less activity in the areas supplied by the circumflex branch than in those supplied by the left anterior descending branch. Fig. 5 shows a comparison between mean epi- and endocardial activity concentrations (per 0.1 mCi injected) of all seven dogs exposed to left coronary artery injection. Each point represents a mean value of about 25 tissue blocks from one heart. The slope of the regression line (b_1) is 0.95 ± 0.08 (S.D.)

activity observed by Wiley *et al.* (1974) after bolus injection of Xe-133 into the left coronary artery. The level of activity varied considerably from one dog to the other (Fig. 2 and Fig. 5). This was probably due to different fractions of the injected activity reaching the left coronary artery and might be explained by different positions of the tip of the catheter used for the injection.

Injection of Xe-133 into the left ventricular cavity (Bagger 1972) resulted in a 15% higher activity in the endo- than in the epicardial part of the wall. The difference was explained by uptake of xenon through the endocardial surface. This explanation is supported by the uniform distribution achieved by the present aortic root injections.

The activity concentrations measured after injection of xenon into the left coronary artery were not different in apical and basal regions supplied by the same main branch of the artery. In some dogs, however, considerably higher activity was found in the area supplied by the left anterior descending branch than in the area of circumflex supply. This non-uniform distribution indicates an insufficient mixing of xenon and blood before branching of the left coronary artery, probably because of the short or even absent main stem of the artery in dogs (Blair 1961). The uneven distribution between areas supplied by the main branches did not influence the ratio of epi- to endocardial activity concentrations, which was identical to that obtained after aortic root injections. These results indicate that the position of the injection catheter is not crucial if injections of tracers are used for estimation of epi- and endocardial flow distribution in dogs. However, its position is critical if dogs are used for regional measurements of flow with scintillation camera, multi-detector systems or autoradiography.

After bolus injection of xenon into the aortic root, the activity transported to each region of the left ventricular wall will be proportionate to flow to that region (Sapirstein 1956). Assuming complete transfer of the isotope from the vascular system to the tissue, a difference in flow between two regions will result in a proportionate difference in initial tissue activities. After injection, during excision of the heart, flow is able to change the activity by wash-out. The amount of activity removed per unit time depends on the volume of distribution of the isotope and the blood flow. The smaller the volume of distribution and/or larger the flow, the faster is the change in tissue activity. In one normal, anesthetized dog the ratio of epi- to endocardial volumes of distribution for xenon was calculated as the ratio of activity concentrations in these regions measured after continuous infusion into the left coronary artery for 8 min. Taubert *et al.* (1972) have shown that myocardial saturation with argon administered by inhalation is obtained in 4-5 min in normal dogs, while Klassen (1972) found saturation time for tritiated water of about 3 min in normal man. Thus, it is probable that myocardial saturation with xenon was attained in the present expt. and that the slight increase in activity recorded externally during the final part of the infusion was due to uptake in extramyocardial tissue, which could be "seen" by the detector. The uniform epi- and endocardial saturation is in accordance with the general assumption of identical volumes of distribution per unit weight (λ partition coefficients). The possibility of diffusion of Xe-133 in and from the tissue before and after freezing of the heart has previously been estimated (Bagger 1972). No change in distribution or amount of activity was observed during 15 min before and about 120 min after freezing. Therefore, the initial ratio of

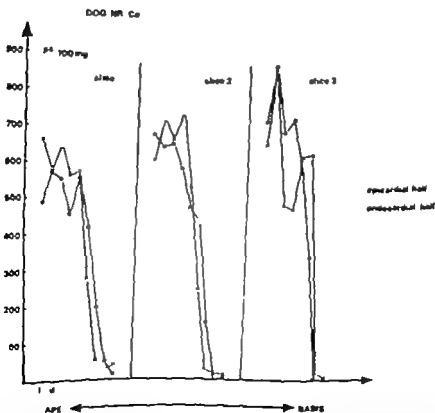
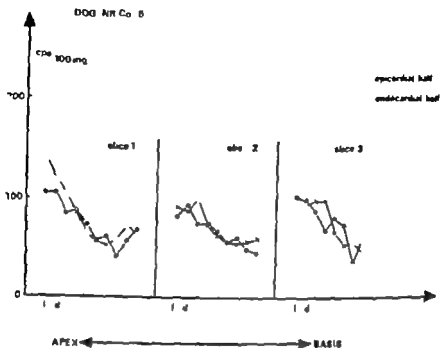


Fig. 3 and 4 The distribution of $Xe-133$ in the apical and endocardial halves of three slices of the left ventricle in two dogs after bolus injection into the left coronary artery plotted in the same way as in Fig. 1

mainly supplies the epicardial half of the left ventricular wall as a consequence of the intramyocardial pressure gradient (Gregg and Eckstein 1941 Brandt and McGroarty 1970, Van Der Meer *et al.* 1970, Armour and Randall 1971). Therefore ep- and endocardial mean flows indicate that diastolic flow is larger and the vascular resistance smaller in the endo- than the epicardial region. This might be due to a different caliber of the resistance vessels supplying the two regions (Provenza *et al.* 1979) and/or a difference in their numbers. Cutarelli and Levy (1963) found a redistribution of Rb-86 in fibrillating dog hearts perfused at a pressure of 100 mmHg with an intraventricular pressure of 5 mmHg. This result does not support the idea of totally different vessels supplying the two regions. Thus the difference in vascular resistance during diastole might be explained by a higher degree of vasodilatation in the endo-epicardial region as suggested by Moir (1972).

Appendix

Assuming that the blood flow per unit weight to the ep- and endocardial regions of the left ventricle (f and f' respectively) remains as initial tissue activities of Xe-133 (A_0 and A'_0) proportional to flow each of the two halves homogeneously perfused, that f and f' are different and that the activity as well as time at each of the two regions (A_0 and A'_0) follow an exponential course (Kety 1949) i.e. after time t , then

$$A = A_0 \exp \left[\frac{-f}{\lambda} t \right] = f^* k \exp \left[\frac{-f}{\lambda} t \right] \quad \text{and}$$

$$A' = A'_0 \exp \left[\frac{-f'}{\lambda} t \right] = f'^* k \exp \left[\frac{-f'}{\lambda} t \right]$$

where k is a constant and λ the partition coefficient between tissue and blood. From these two equations it follows that

$$\ln f = \ln f^* - \ln \lambda - \ln \frac{A}{A_0} + \ln f$$

From values of f^* , t and λ this equation can be written

$$y = f^* + q \quad (1)$$

where the constant $q = \ln \lambda$ and the constant $q = -\ln f^* / \lambda - \ln (A_0 / A) + \ln f$

Graphical solution of equation (1) f^* can be determined as the abscissa to the point of intersection between the lines $y = \ln f^*$ and $y = f^* + q$.

A previously determined mean ratio of ep- to endocardial activity concentrations (A_0/A'_0) was 0.98 ± 0.05 (S.E.). Therefore the probability that the true mean ratio of the population exceeds 1.10, is less than 1%. Assuming that true $A_0/A'_0 = 1.10$, $\lambda = 0.7$ $f = f'$ and $f' = 1$ ml g⁻¹ min⁻¹ = 0.25 area (the time interval between injection of Xe-133 and completion of heart suction) f^* will be 0.87 ml g⁻¹ min and $f = A_0/A'_0 = 1.15$. This means that at normal coronary blood flow the initial ratio of activity concentrations will be less than 5% higher than the value measured 15 s later. Assuming $f' < f$ the other answer of equation (1) would be $f' = 6.5$ ml g⁻¹ min which is most unlikely from hemodynamic point of view and incompatible with the smallness of the scatter of the observed values of A_0/A'_0 relative to the size of the y -values.

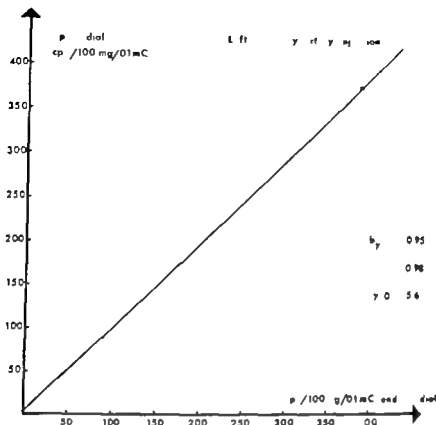


Fig 5 Comparison of mean epi- and endocardial activity (cps/100 mg/0.1 mCi injected) of the left ventricular wall of 7 dogs after intracoronary bolus injection.

epi- to endocardial activity concentrations could only be changed by different epi- and endocardial blood flows in the period used for excision of the heart.

It is shown in the appendix that a normal epicardial blood flow of 1 ml g⁻¹ min⁻¹ during a period of 15 s after injection of Xe-133 will result in a reduction of the initial ratio of epi- to endocardial activity concentrations of less than 5%. This modest reduction due to washout of activity corresponds to the lack of correlation between activity ratios and excision time in the individual experiments. Thus, the measured distribution of activity in the left ventricular wall presumably represents a reliable picture of the flow distribution.

In summary the study has shown that adequate mixing of xenon and left coronary artery blood is obtained by aortic root injections: that blood flow is uniform throughout the left ventricular free wall and that intracoronary injections of tracer can result in a false picture of different flows to the left anterior descending and circumflex supplied areas, due to inadequate mixing, but may be used for estimations of epi- to endocardial flow ratio.

The uniform epi- and endocardial distribution of flow is in accordance with results obtained by infusion of other diffusible tracers (Cutarelli and Levy 1963, Palmer-Fam and McGregor 1966, Moir and DeBra 1967, Griggs and Nakamura 1968) and with some of the results obtained with small radioactive microspheres (7–15 μ m) (Becker, Fortuin and Pitt 1971, Buckberg *et al.* 1972 and 1975, Becker, Ferreira and Thomas 1974, Flixler, Saunders and Sugg 1974). Downey and Kirk (1974) have shown that systolic coronary blood flow

dominantly supplies the epicardial half of the left ventricular wall as a consequence of systemic isometry-cardial pressure gradient (Gregg and Eckstein 1941 Brandt and McGrew 1948, Beid *et al.* 1970, Van Der Meer *et al.* 1970 Armour and Randall 1971). There is, without exception, evidence that diastolic flow is larger and diastolic vascular resistance smaller in the endo- than the epicardial region. This might be explained by a different caliber of the resistance vessels supplying the two regions (Provenza and Scheraga 1959) and/or a difference in their numbers. Cutarelli and Levy (1963) found a uniform distribution of Rb-86 in fibrillating dog hearts perfused at a pressure of 100 mmHg and with an intraventricular pressure of 5 mmHg. This result does not support the idea of anatomically different vessels supplying the two regions. Thus the difference in vascular resistance during diastole might be explained by a higher degree of vasodilatation in the endo- than epicardial region as suggested by Moir (1972).

Appendix

Assuming that the blood flow per unit weight to the epi- and endocardial regions of the left ventricular wall (f' and f'' respectively) results in linear tissue activities of Xe-133 (A'_t and A''_t) proportionate to flow as each of the two halves is homogeneously perfused, that f' and f'' are different and that the activity as a function of time in each of the two regions (A'_t and A''_t) follows an exponential course (Kety 1949) at least in the first 15 s, then

$$A'_t = A' \exp \left[\frac{-f'}{\lambda} t \right] = f' k \exp \left[\frac{-f'}{\lambda} t \right] \quad \text{and}$$

$$A''_t = A'' \exp \left[\frac{-f''}{\lambda} t \right] = f'' k \exp \left[\frac{-f''}{\lambda} t \right]$$

where k is a constant and λ the partition coefficient between tissue and blood.

From these two equations it follows that

$$\ln f' = \lambda \ln A'_t - \lambda \ln A''_t = \ln \frac{A'_t}{A''_t} + \ln f''$$

For given values of f' and λ this equation can be written

$$\ln f' - \ln f'' = q \quad (1)$$

where the constant q and the constant λ is $q = \lambda \ln (A'_t/A''_t) + \ln f''$.

By graphical solution of equation (1) f' can be determined as the y -intercept to the point of intersection between the functions $y = \ln f'$ and $y = \ln f'' + q$.

The experimental determined mean ratio of epi- to endocardial activity concentrations (A'_t/A''_t) was 0.98 ± 0.15 (S.E.). Therefore the probability that the true mean ratio of the population exceeds 1.10, is less than 5%.

Assuming that true $A'_t/A''_t = 1.10$, $2-0.7 f' > f''$ and $f'' = 1 \text{ ml g}^{-1} \text{ min}^{-1}$ as 0.25 mm (the true interval between injection of Xe-133 and completion of heart section) f' will be 0.87 ml g min^{-1} and $f'/f'' = 0.87$. This means that at normal coronary blood flow the initial ratio of activity concentrations will be less than 5% higher than the value measured 15 s later. Assuming $f' = f''$ the other member of equation (1) would be $f' = 6.5 \text{ ml g}^{-1} \text{ min}^{-1}$ which is most unlikely from hemodynamic point of view and incompatible with the magnitude of the scatter of the observed values of A'_t/A''_t relative to the scatter of the f -values.

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Evidence for a Rate-Sensitive Regulatory Mechanism in Myogenic Microvascular Control

By

PER-OLOF GRÄNDE, JAN LUNDAVALL and STEFAN MELLANDER

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Abstract

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To reveal a possible rate-sensitive component in the myogenic control, changes of total and segmental vascular resistances in sympathectomized skeletal muscle in response to alteration of vascular transmural pressure (extravascular pressure) by 40 mmHg were compared when the pressure change was applied at two distinctly different rates (15 and 170). The papaverine-dilated vascular bed showed an entirely passive behaviour whereas the normal, myogenically reactive vascular bed responded with active constriction upon transmural pressure increase and active dilation upon pressure decrease. These responses are especially pronounced in the microvessels where a clearcut two-component effector response was observed. The magnitude of the initial component was distinctly correlated to the rate at which the transmural pressure stimulus was applied, whereas the later steady state component during the static pressure change was rate-independent. At the high rate of pressure increase, the initial rate-dependent microvascular constriction response was some ten times larger than the steady state response. These observations indicate the existence of a rate-sensitive as well as a static component in the myogenic response to changed transmural pressure, an interpretation strongly supported by previous analogous study on isolated single-unit vascular smooth muscle (Johansson and Mellander 1975). It is concluded that the microvessels in skeletal muscle are highly responsive to myogenic stimuli and that emphasis should be placed on the dynamic rather than the static characteristics of the stimulus. Such rate-sensitivity in myogenic control would seem to facilitate prompt and proper vascular adjustments, for instance in myogenic autoregulation.

The Bayliss hypothesis (1902) of enhanced contractile activity in vascular smooth muscle in response to increased blood pressure has received strong experimental support in recent years from hemodynamic whole organ and vital microscopy studies (e.g. Folkow 1949 1964 Wiedeman 1963 Mellander *et al.* 1964 Johnson and Wayland 1967 Baerz *et al.* 1974). The described active myogenic responses to changes in vascular transmural pressure have been considered of great importance for various autoregulatory phenomena in the local circulatory control, and myogenic mechanisms may also be a major determinant for the development of normal basal vascular tone.

Myogenic reactivity has so far been studied almost exclusively in terms of the active responses which develop upon a given steady state increase or decrease in vascular trans-

1 Correspondence to this author

response, little attention being paid to the possible effect of the rate at which pressure and length steady state effects might thus be considered to be the result mainly of a myogenic stimulus related to the amplitude of the pressure change. Some recent studies, however, provided circumstantial evidence for the presence of an additional, rate-sensitive component in the myogenic control by the observation of in-vascular tone upon shift from steady to artificial sinusoidal pressure perfusion (Wedin 1969) or from steady to normal pulsatile perfusion (Mellander and Arvidsson 1974). Direct evidence for a rate-sensitive component in the vascular myogenic response to changes in length was later obtained from *in vitro* observations of electrical and mechanical activity in the rat portal vein (Johansson and Mellander 1975) where the muscle was exposed to a given static stretch or shortening applied at various rates over the range between -3 and $+3\%$ of the muscle length/s. Static stretch by 40% of the length evoked moderate excitatory effects in terms of a 10 to 15% increase in spike frequency above the resting control value and a corresponding increase in active force. A static stretch was much more effective causing graded excitatory responses which at the rates of stretch were up to 20 times more pronounced than those evoked by static stretch. Passive shortening at different rates caused graded inhibitory responses below the resting level, complete inhibition of electrical and mechanical activity being observed at the negative test value of dl/dt .

In view of current observations, the myogenically active vascular smooth muscle may be considered to act as a mechanoreceptor-mechanoeffector unit in which deformation (strain) has effects on a pacemaker mechanism, causes facilitation of spike discharge spread to the neighbouring muscle effector cells. The net effect of such mechanical coupling in response to receptor deformation is changes in the frequency of spike discharge and attendant active changes in vascular tone. The mentioned *in vitro* observations have indicated that myogenic control is dominated by the dynamic rather than the static features of the pacemaker stimulus, but before this new dimension in the concept of myogenic regulation is adopted more universally the principle should be more firmly tested in the *in vivo* situation as well.

In the present study an attempt was made to test this hypothesis on the sympathetomized rat bed of skeletal muscle by exposing the vessels to increases and decreases in transmural pressure applied at distinctly different rates. Observations of total vascular resistance, peripheral resistances in large arterial vessels, microvessels, and large veins were made during the period of plastic change of transmural pressure and also in the steady state phase of constant increased or decreased pressure. The results support the concept of an important, rate-sensitive component in the myogenic control with special influence on the regulatory resistance vessels *in vivo*. Preliminary reports of this work have been presented here (Mellander, Lundvall and Grönroos 1976, Mellander 1976, a, b).

Methods

Male rats of both sexes, weighing $28-40$ kg, were used in the study. The animals were anaesthetized with sodium pentobarbital (30 mg/kg b.w.) and urethane (100 mg/kg b.w.) after induction with ether. Body temperature was maintained at $36 \pm 0.5^\circ\text{C}$ throughout the experiment.

The investigation was performed on the acutely denervated vascular bed of the right lower leg muscle which were isolated so that the popliteal artery and vein formed the sole vascular connections with the rest of the body (for details see Lundvall 1972). In brief, the skin was dissected free and removed from the lower leg and its muscles were separated completely from the thigh muscles. The paw was removed at the ankle. After heparinization (750 I U/kg b.wt.), blood flow was diverted from the ipsilateral femoral artery to the popliteal artery through a short shunt circuit and the popliteal vein was cannulated and its outflow shunted via tubes to a funnel connected to the right jugular vein. Mean arterial inflow of blood was recorded continuously with an electromagnetic flowmeter (Biotronex) or more frequently with the use of the pressure gradient flowmeter principle. For the latter purpose, a differential manometer (Narco Bemic LX1601D) was used for monitoring the pressure drop across a fixed length of the arterial shunt. The diameter of the shunt was chosen so as to keep the pressure drop small (< 10 mmHg), yet permitting reliable determination of even minute variations of the pressure drop and, hence, blood flow. A series of pilot tests of this flowmeter device demonstrated that it permitted accurate and reproducible flow determinations over the range encountered in the present experiments. Venous outflow of blood was concomitantly measured using an optical drop recorder inserted in the venous shunt circuit. Mean arterial inflow pressure (below denoted AP) and venous outflow pressure (VP) were measured from T-tubes close to the cannulated popliteal artery and vein. Pressures from a small intramuscular arterial vessel ("small artery pressure" SAP) and from a small venous vessel ("small vein pressure" SVP) were monitored as well. For this purpose, the small artery and vein on the posterior surface of the gastrocnemius muscle were ligated proximally with flexible nylon tubing (d. of tip ~ 0.4 mm) filled with heparinized saline solution were inserted distally. In all ligations test experiments showed that an induced pressure change was transmitted with a measurable delay through these cannulas. With this technique, the pressure in small vessels within the muscle is transmitted via collaterals to the cannulated vessels, a principle for measurement of pressures in the microcirculation originally described by Haddy *et al.* (1934). Frequent checks throughout the experiment ensured free communication between the intramuscular vessels and the cannulas: the small artery and vein (see Lundvall 1972). Statam pressure transducers were used for the pressure measurements.

After the vascular cannulations were completed, the femur was divided and plugged and the muscle preparation was placed in a fixed position in a temperature controlled plethysmograph (37°C), filled with Tyrode solution. The entrance of the plethysmograph, through which the arterial and venous cannulas passed, was hermetically sealed with flanges and curing silicone rubber (Medical Elastomer Dow Corning). The fluid in the plethysmograph was in communication via a polyethylene tubing with a reservoir placed outside the plethysmograph (for details see Grände *et al.* 1974). The hydrostatic fluid pressure inside the plethysmograph, measured at the midlevel of the muscle region with a Statam transducer, could be adjusted to desired levels by varying the height of the reservoir in relation to the muscle preparation. The reservoir was connected to an electronic gravimetric transducer (Grass FT 10) so that fluid displacement between the plethysmograph and the reservoir caused by changes of tissue volume, could be followed continuously. In the resting control state, the pressure in the plethysmograph was adjusted to the atmospheric zero level. VP in the control period was adjusted to a level (4–7 mmHg), giving a constant tissue volume (isovolumic state) and was maintained at this level throughout the experiment. A transcapillary fluid equilibrium was thus achieved in the control period. By lowering the reservoir the pressure in the plethysmograph and hence tissue pressure, could be decreased. In this way all parts of the vascular bed of the studied muscle region were exposed to a standardized increase of vascular transmural pressure (40 mmHg) applied in two different rates (in 15 and 120 s). Return of the reservoir to the zero level permitted graded decline of vascular transmural pressure at the same standardized rates. All measured parameters were recorded on a Grass polygraph.

With this experimental approach changes of total vascular resistance and of segmental resistances in the muscle region evoked by the induced alteration of vascular transmural pressure could be followed (cf. Lundvall 1972). From observed alterations of tissue volume the induced changes of regional blood content (capacitance responses) and transcapillary fluid movements could be determined well (see Mellander *et al.* 1964; Lundvall *et al.* 1967). Total vascular resistance in the muscle region was calculated $(AP - VP) / Q$, where Q is arterial flow. The segmental resistances were determined in the following way: Proximal arterial resistance $(AP - SAP) / Q$, arterial flow. Microvascular resistance $(SAP - SVP) / Q$, arterial flow (neglecting the minor effect of net transcapillary fluid transfer on blood flow through the exchange and small capillary vessels). And "large vein resistance" $(SVP - VP) / Q$, venous outflow. Venous outflow (or arterial flow minus recorded capacitance effects and net fluid filtration) was used for the latter calculation, since transcapillary fluid movements and transient capacitance effects were induced by changed transmural pressure, implying some difference in the volume flow in arterial and venous vessels under these conditions.

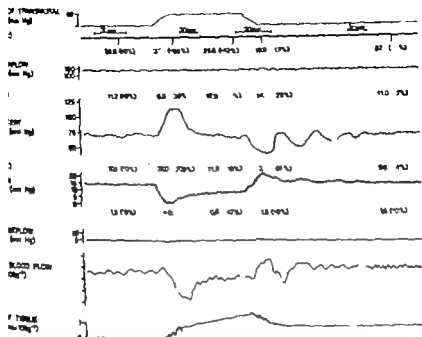


FIGURE 1. Changes in total and segmental resistance in the myogenically responsive vascular bed of skeletal muscle evoked in response to increased and decreased arterial transmural pressure (100 mmHg) applied at high rate (2.7 mmHg/s). Note the pronounced macrovascular (R_{macro}) and diastolic responses during the periods of phasic increase and decrease in transmural pressure and moderate constrictor response during the period of constant, increased transmural pressure. These data indicate an important rate-sensitive component and less pronounced "static" or myogenic control. The arrows below the time scale depict the time at which resistance is 1. (Paper speed: 10 mm/sec during expt. as indicated in time scale.)

Results

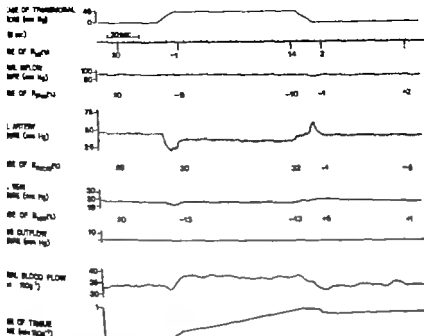
The first experiment was made in the present study to investigate whether the resistance vessels are myogenically responsive not only to the magnitude of a vascular transmural pressure increase or decrease ("static" steady state response) but also to the rate of the change ("dynamic" response). For this purpose changes of skeletal muscle vascular resistance in response to alteration of vascular transmural pressure (extravascular pressure) were compared when the pressure change was applied at two distinctly different rates, i.e., 15 and 125 mmHg/s. Observations were made under conditions of negligible adrenergic activity or on the normal, myogenically reactive, as well as on the passive, myogenically insensitive, vascular bed.

Figure 1 shows original recordings from an experiment in which the "normal" vascular bed was subjected to a rapid change in transmural pressure (about 2.7 mmHg/s). Calculated values for total vascular resistance (R_{total}), and for segmental resistances, i.e., "proximal arterial resistance" (R_{prox}), microvascular resistance (R_{micro}), and "venous resistance" (R_{ven}) are plotted in the figure (for definitions see Methods and Discussion). In the control period, arterial transmural pressure was adjusted to the normal level which established a trans-

The investigation was performed on the acutely denervated vascular bed of the right lower leg muscle which were isolated so that the popliteal artery and vein formed the sole vascular connections with the rest of the body (for details see Lundvall 1977). In brief the skin was dissected free and removed from the lower leg and its muscles were separated completely from the thigh muscles. The paw was removed at the ankle. After heparinization (750 I.U./kg b.wt.), blood flow was diverted from the ipsilateral femoral artery to the popliteal artery through a short shunt circuit and the popliteal vein was cannulated and its outflow shunted via tubes to a funnel connected to the right jugular vein. Mean arterial inflow of blood was recorded continuously with an electromagnetic flowmeter (Blotronex) or more frequently with the use of the pressure gradient flowmeter principle. For the latter purpose, a differential manometer (Narco Semic. LX1601D) was used for monitoring the pressure drop across a fixed length of the arterial shunt. The diameter of the shunt was chosen so as to keep the pressure drop small (< 10 mmHg), yet permit reliable determination of even minute variations of the pressure drop and hence, blood flow. A series of pilot tests of this flowmeter device demonstrated that it permitted accurate and reproducible flow determinations over the range encountered in the present experiments. Venous outflow of blood was concomitantly measured using an optical drop recorder inserted in the venous shunt circuit. Mean arterial inflow pressure (below denoted AP) and venous outflow pressure (VP) were measured from T-tubes close to the cannulated popliteal artery and vein. Pressures from a small intramuscular arterial vessel ("small artery pressure" SAP) and from a small venous vessel ("small vein pressure" SVP) were monitored as well. For this purpose the sural artery and vein on the posterior surface of the gastrocnemius muscle were ligated proximally and flexible nylon tubings (I.D. of tip ~ 0.4 mm) filled with heparinized saline solution were inserted distal to the ligations. Test experiments showed that an induced pressure change was transmitted with no measurable delay through these cannulas. With this technique, the pressure in small vessels within the muscle is transmitted via collaterals to the cannulated vessels, a principle of measurement of pressures in the microcirculation originally described by Haddy *et al.* (1954). Frequent checks throughout the experiment ensured free communication between the intramuscular vessels and the cannulas, the sural artery and vein (see Lundvall 1972). Statham pressure transducers were used for the pressure measurements.

After the vascular cannulations were completed, the femur was divided and plugged and the muscle preparation was placed in a fixed position in a temperature controlled plethysmograph (37°C), filled with Tyrode's solution. The entrance of the plethysmograph, through which the arterial and venous cannulas passed, was hermetically sealed with flanges and curing silicone rubber (Medical Elastomer, Dow Corning). The fluid in the plethysmograph was in communication via a polyethylene tubing with a reservoir and placed outside the plethysmograph (for details see Grände *et al.* 1974). The hydrostatic fluid pressure inside the plethysmograph, measured at the midlevel of the muscle region with a Statham transducer, could be adjusted to desired levels by varying the height of the reservoir relative to the muscle preparation. The reservoir was connected to an electronic gravimetric transducer (Grass FT 10) so that fluid displacements between the plethysmograph and the reservoir caused by changes of tissue volume, could be followed continuously. In the resting control state, the pressure in the plethysmograph was adjusted to the atmospheric zero level. VP in the control period was adjusted to a level (4–7 mmHg), giving a constant tissue volume (saw lumic rate) and was maintained at this level throughout the experiment. A transcapillary fluid equilibrium was thus achieved in the control period. By lowering the reservoir the pressure in the plethysmograph, and hence tissue pressure, could be decreased. In this way 11 parts of the vascular bed of the studied muscle region were exposed to a standardized increase of vascular transmural pressure (40 mmHg) applied at two different rates (in 15 and 120 s). Return of the reservoir to the zero level permitted graded declines of vascular transmural pressure at the same standardized rates. All measured parameters were recorded on a Grass polygraph.

With this experimental approach, changes of total vascular resistance and of segmental resistances in the muscle region evoked by the induced alteration of vascular transmural pressure could be followed (cf. Lundvall 1972). From observed alterations of tissue volume the induced changes of regional blood content (capacitance responses) and transcapillary fluid movements could be determined as well (see Mellander *et al.* 1964, Lundvall *et al.* 1967). Total vascular resistance of the muscle region was calculated as $(AP \cdot VP) / \text{arterial flow}$. The segmental resistances were determined in the following way: "Proximal arterial" resistance = $(AP - SAP) / \text{arterial flow}$. Microvascular resistance $(SAP - SVP) / \text{arterial flow}$ (neglecting the minor effect of net transcapillary fluid transfer on blood flow through the exchange and small exchange vessels). And "large vein" resistance = $(SVP - VP) / \text{venous outflow}$. Venous outflow (or arterial inflow minus recorded capacitance effects and net fluid filtration) was used for the latter calculation, since transcapillary fluid movements and transient capacitance effects were induced by changed transmural pressure, implying some difference in the volume flow in arterial and venous vessels under these conditions.



Similar exp't to that in Fig. 1 performed on the papaverine-dilated mouse vascular bed. Note the passive behavior of all resistance sections in response to changed transmural pressure in this exp't. Active reactivity was abolished.

for the venous one. For reasons discussed below this active constrictor response in all good is essentially myogenic in nature. This constrictor mechanism, however, was much effective during the phase of the transmural pressure change than in the later phase of rise, increased pressure, which might indicate the presence of an initial rate-sensitive later static component in the myogenic vascular control. If this interpretation is correct, would expect the rate-sensitive component of the myogenic response to be less pronounced if the myogenically reactive vascular bed was exposed to the same transmural pressure change, *i.e.* 40 mmHg, but applied at a significantly lower rate than in Fig. 1. Fig. 3 shows original recordings from the same preparation as in Fig. 1 but the vascular bed was exposed to low rate transmural pressure change (0.33 mmHg/s). It can be seen that the pattern of vascular response in essential parts was qualitatively similar to that induced by the rapid transmural pressure change in Fig. 1 but that the responses during the pressure change, as predicted above, were quantitatively much less pronounced. Thus, the maximum response, *i.e.* at the completion of the pressure rise, R_{ven} was now raised by only 23% and R_{artery} by only 55% above the values in the control period compared to 63% and 208% in Fig. 1. $R_{\text{p_{aorta}}}$ in Fig. 3 was virtually unchanged at this time, as the venous compartment showed roughly the same degree of passive dilation as in Fig. 1. The vascular responses in the steady static phase of elevated transmural pressure, on the other hand, were practically identical in Fig. 1 and 3. When transmural pressure was raised at the low rate of 0.33 mmHg/s (Fig. 3), the vascular effects were similarly much

capillary Starling fluid equilibrium as indicated by the isovolumic tissue volume con-
 Control pressures and blood flow were constant except for minor normal cyclic variation.
 Arterial inflow pressure and venous outflow pressure were not significantly affected by the
 subsequent changes of transmural pressure but other marked vascular reactions were induced.
 Thus, during the 15 s period of transmural pressure rise, blood flow first showed
 transient slight increase, but within few seconds it decreased rapidly to attain a minimum
 value very shortly after the completion of the pressure rise. These observations demonstrate
 a transient passive dilation followed quickly by a constrictor response of the resistance
 vessels, which increased total vascular resistance to a level about 65% above that in the
 control period. The segmental resistances also changed markedly as evidenced by the concomitant
 changes of flow, SAP and SVP. Thus, few seconds after the beginning of the
 transmural pressure rise, SAP increased and SVP decreased rapidly, maximal changes being
 reached just after completion of the transmural pressure rise. At this time microvascular
 resistance had increased by about 210% above the control level, whereas proximal arterial
 resistance had decreased by almost 40% and there was also a fall of venous resistance
 initially to quite a low level. Some 10 s after the level of constant, high transmural pressure
 was reached, blood flow and SVP again rose and SAP decreased gradually to attain new
 steady state values within about two minutes. At this time, R_{tot} and R_{micro} were only moderately
 increased (by 17 and 18% above control), whereas R_{ven} stabilized at a level some
 50% below the control value. R_{prox} had now increased slightly above the control level (15%).

During the subsequent decrease of transmural pressure back to the control level at a rate
 of -2.7 mmHg/s (Fig. 1) the pattern of vascular response was opposite to the one during
 the pressure rise, insofar that blood flow and SVP increased and SAP decreased rapidly.
 At completion of the pressure fall R_{tot} and R_{micro} were significantly lower than in the initial
 control period, *i.e.* by 13% and 61% respectively, indicating clearcut dilator effects. R_{prox}
 at the same time had further increased somewhat, whereas R_{ven} approached the control
 value. Some 10 s after the return to the control transmural pressure level, blood flow and
 SVP again decreased and SAP increased markedly, after which followed an oscillatory
 behaviour gradually damped out in 2-3 min when control values for blood flow, R_{tot} ,
 R_{prox} , R_{micro} and R_{ven} were reached.

Fig. 2 shows the effects in the maximally dilated vascular bed of the same transmural
 pressure alterations as in Fig. 1. Vascular tone and myogenic reactivity in this preparation
 was abolished throughout the observation period by a close arterial infusion of a large dose
 (100 mg/kg tissue) of papaverine. It can be seen that this vascular bed showed a passive
 behaviour in all resistance sections as indicated by the calculated figures for the per cent
 decrease in total, proximal arterial, microvascular and venous resistance below control
 during the transmural pressure increase, and the quite rapid return to control resistance
 values upon the subsequent pressure decline.

An increase of transmural pressure in the maximally dilated vascular bed (Fig. 2) thus
 caused the expected effects of passive vessel distension in all resistance sections, whereas the
 vascular bed with normal tone (Fig. 1) showed clear signs of some mechanism which effectively
 counteracted such distension and even caused pronounced constriction in all sections.

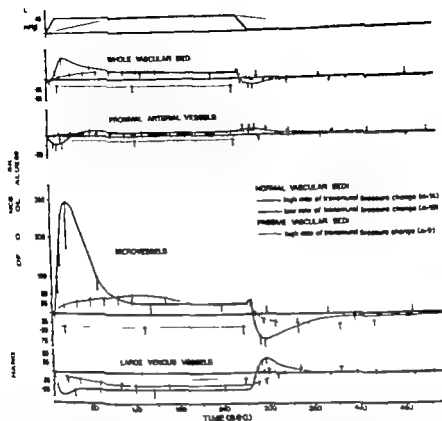


Fig. 4. Coupled data for total and segmental resistance changes in the normal, myogenically reactive and the passive, myogenically inactive vascular bed of skeletal muscle evolved in response to increased and decreased vascular transmural pressures (40 mmHg applied at high (2.7 mmHg/s) and low (0.33 mmHg/s) rates) to the symbols see key number of observations. Note the presence of both rate-dependent and T -independent (steady state) resistance responses in the microvessels of the normal vascular bed and the active behavior of the myogenically inactive vascular bed. (The period of constant high transmural pressure lasted in all experiments about 2.5 min, implying some extrapolation in time of the steady state values depicted by the solid base curves.)

resistance in all vascular sections during the whole period of increased transmural pressure. In the vascular bed with preserved myogenic tone (solid and dashed curves), a passive distension effect of the transmural pressure rise was maintained only in the venous section but was very transient in the others. Thus, within few seconds, resistance exceeded the control value in the overall resistance vessels and the microvessels and, with some longer delay this also occurred in the proximal arterial vessels, indicating active constrictor responses in these sections during raised transmural pressure. In the overall resistance vessels and in the microvascular section, the constrictor responses reached clearcut peak values just after completion of the transmural pressure rise. However the magnitudes of these constrictor responses were much greater with the high (2.7 mmHg/s) than the low (0.33 mmHg/s) rate of the transmural pressure increase. The later steady state constrictions (reached about 2 min after

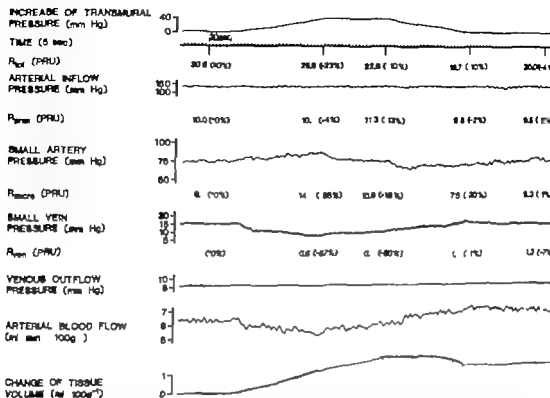


Fig. 3. Experiment illustrating total and segmental resistance changes in the sympathectomized myogenically reactive vascular bed of skeletal muscle evoked in response to increased and decreased arterial transmural pressure (40 mmHg) applied at low rate (0.33 mmHg/s). Note the dominance of the "rate-sensitive" but not of the static component in the microvascular myogenic resistance response compared with the resistance effect at high rate of pressure change shown in Fig. 1.

less pronounced than during the rapid pressure decline (Fig. 1) recovery to control vascular tone after the pressure decline was achieved in both cases within 2–3 min, but in Fig. 3 without the pronounced oscillatory behaviour exhibited after the rapid transmural pressure decline.

Fig. 4 summarizes the results from similar expts. performed on 11 skeletal muscle preparations which, after completed surgery from a technical point of view could be considered satisfactory. The data are given in terms of total and segmental vascular resistance changes expressed in per cent of the control values prevailing before the transmural pressure increase (cf Fig. 1–3). The key in Fig. 4 defines the three different types of experiments and also the number of aortal observations (n). The data in the diagrams refer to mean values \pm S.D. and the curves connecting the different points are fitted by eye and smoothed out neglecting minor resistance variations between the different points. Table 1 shows the mean PRU values \pm S.D. for total, proximal arterial, microvascular and venous resistances in the initial control period (corresponding to time zero in Fig. 4) for the three different types of expts. It can be seen from Fig. 4 that, for the whole material, the three patterns of resistance response to changed transmural pressure much resembled those already described (Fig. 1–3). The following main conclusions may be drawn. The vascular bed with abolished tone (dotted curve) showed an entirely passive behaviour (distension) in terms of decreased

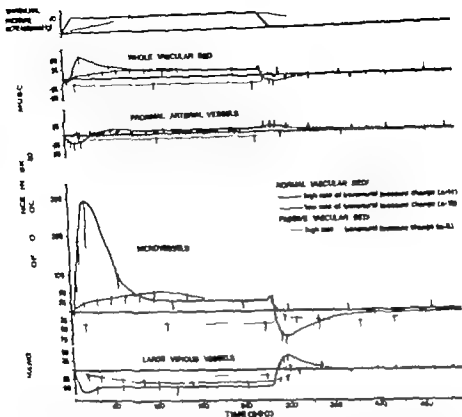


Fig. 4 Coupled data for total and segmental resistance changes in the normal, myogenically reactive and passive, myogenically inactive vascular bed of skeletal muscle evoked in response to increased and decreased vascular transmural pressure (40 mmHg) applied at high (2.7 mmHg/h) and low (0.33 mmHg/h) rates (for symbols see key - similar of observations). Note the presence of both rise-dependent and rise-independent (steady state) resistance responses in the microvessels of the normal vascular bed and the passive behavior of the myogenically inactive vascular bed. (The period of constant high transmural pressure lasted in all experiments about 2.5 min, implying some extrapolation in time of the steady state data depicted by the solid line curves.)

resistance in all vascular sections during the whole period of increased transmural pressure. In the vascular bed with preserved myogenic tone (solid and dashed curves), a passive distension effect of the transmural pressure rise was maintained only in the venous section but was very transient in the others. Thus, within few seconds, resistance exceeded the control value in the overall resistance vessels and the microvessels and, with some longer delay, this also occurred in the proximal arterial vessels, indicating active constrictor responses in these sections during raised transmural pressure. In the overall resistance vessels and in the microvascular section, the constrictor responses reached clearcut peak values just after completion of the transmural pressure rise. However the magnitudes of these constrictor responses were much greater with the high (2.7 mmHg/h) than the low (0.33 mmHg/h) rate of the transmural pressure increase. The later steady state constrictions (reached about 2 min after

TABLE 1 Mean control values \pm S.D. for total and segmental vascular resistances (PRU mmHg per 100 g tissue⁻¹) in skeletal muscle in the three types of experiments (illustrated in Fig. 4).

Control PRU	Normal vasc. bed High rate pressure change	Normal vasc. bed Low rate pressure change	Passive vasc. bed High rate pressure change
R_{tot}	19.0 ± 6.4	17.6 ± 6.1	4.3 ± 1.4
R_{prox}	11.6 ± 3.9	10.3 ± 3.1	3.1 ± 1.4
R_{micro}	6.4 ± 3.7	6.4 ± 2.8	0.6 ± 0.4
R_{ven}	1.0 ± 0.7	0.9 ± 0.8	0.6 ± 0.4

the peak) on the other hand, were about equal at both rates. In the proximal arterial vessels the constriction was slower in onset and it raised resistance in the steady state to a level just barely above the control value.

When transmural pressure was decreased in the vascular bed with abolished tone, a vascular sections showed a passive behaviour the resistances quickly returning to control values after a transient overshoot. In experiments with preserved vascular tone, such behaviour was noted in the venous and to some extent also in the proximal arterial sections whereas the overall and microvascular resistance vessels exhibited clearcut active dilator responses in terms of significantly decreased resistance compared to the preceding and also to the control values. Note that these dilator responses, reaching their maxima soon after the completed pressure decline, were much more pronounced with high than low rate of pressure decrease. Few minutes later all resistances had returned to their initial control levels.

The same type of expt. as depicted by the solid line in Fig. 4 was performed on 3 of the cats before and after complete α -adrenoceptor blockade with phenoxylbenzamine (15 mg kg⁻¹ muscle tissue). The effectiveness of the blockade was tested with a large dose (10 μ g kg⁻¹ muscle tissue) of noradrenaline given close arterially. The vascular constrictor effect of increased transmural pressure were practically identical before and after the blockade indicating that they were not mediated by an α -adrenergic mechanism.

The results described so far seem to strongly indicate the presence of a rate-sensitive ("dynamic") component in the myogenic vascular control causing excitatory (constrictor) responses, especially in the microvessels, during the phase of increasing transmural pressure and inhibitory (dilator) responses during the phase of the pressure decline. The magnitude of the dynamic response was graded in relation to the rate of the pressure change: further its onset occurred within few seconds, and it seemed to be fully developed within 15–20 s irrespective of the rate of the pressure change (see Fig. 1, 3 and 4). The data also support the view of a "static" component in the myogenic control apparently related to the magnitude of the transmural pressure increase (see below) and revealed as a moderate steady state constrictor response in the microvessels and the proximal arterial vessels during the phase of constant, increased pressure. The results might also warrant the conclusion that the microvessels are more sensitive to "dynamic" than "static" myogenic stimuli, whereas the proximal arterial vessels may be dominated in their control by the latter type. Large venous vessels, on the other hand, seem to be little, if at all, controlled by myogenic mechanisms.

It should be emphasized that our whole material consisted of 14 cats, but, after completed surgery 3 preparations were considered unsatisfactory because of technical failure, such as hypothermia or major accidental bleedings, leading, among other things, to arterial hypotension well below the normal level. It is well known from previous studies (e.g. Folkow 1949, Lohkall *et al.* 1967) that such preparations are largely devoid of myogenic reactivity—a fact which was confirmed on these three preparations. It was therefore considered justified to exclude results from these experiments in the material presented in Fig. 4, but still they could be reported. These preparations, in contrast to the others, characteristically showed quite marked dilation in all resistance sections during the phase of the transmural pressure increase, but there was a slight tendency of gradual return of resistance towards the control level in the proximal arterial vessels and in the microvessels in the following steady state phase of constant, increased pressure. It thus appears that these preparations were largely insensitive to myogenic stimuli, at least to "dynamic" ones. Whatever the explanation of the failing reactivity these experiments can help to exclude the possibility that rheological effects of transcapillary plasma fluid loss by viscosity changes or by sludging could have contributed to the vascular resistance changes in the reactive vascular bed (Fig. 4). Since the typical resistance increase depicted in this figure was lacking in these three preparations despite the fact that fluid filtration ($0.75 \text{ ml min}^{-1} 100 \text{ g}^{-1}$) as well as blood flow were in the same order of magnitude in all experiments, rheological factors could hardly have been responsible for the results presented in Fig. 4.

Discussion

Local intrinsic vascular regulation is considered to be accomplished mainly by the myogenic and the chemical/metabolic control systems (e.g. Mellander and Johansson 1968, Johnson 1974). There is much evidence to indicate that both these mechanisms by interaction are importantly involved in autoregulatory phenomena, such as autoregulation of blood flow (e.g. Folkow 1962, Berne 1964, Johnson 1964) and of capillary hydrostatic pressure (e.g. British and Mellander 1974).

For a specific study of myogenic reactions to changed transmural pressure, any significant alteration of blood flow must be avoided, since this leads to secondary interference with the metabolic control system. Observations of myogenic responses to changes in arterial inflow pressure or ~~excess~~ outflow pressure are therefore quite difficult to interpret because of the attendant variation of flow. With the present plethysmographic approach by which the whole vascular bed could be exposed to a standardized change of vascular transmural pressure by alteration of extravascular pressure, such problems were largely overcome. Strong and precisely graded transmural pressure increases or decreases could thus be applied without affecting the arterio-venous pressure difference. Hence, blood flow was not primarily altered, except for the effects of passive vessel distension or passive elastic recoil, but such flow effects, as shown by the results (Fig. 1 and 3), were almost negligible. It is therefore believed that influences from the metabolic control system can be largely disregarded in the interpretation of the described constrictor responses evoked by the transmural pressure increase and the dilator responses evoked by the pressure decrease. Since autonomic nervous

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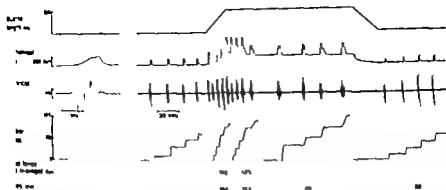
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the peak) on the other hand, were about equal at both rates. In the proximal arterial vessels the constriction was slower in onset and it raised resistance in the steady state to a level barely above the control value.

When transmural pressure was decreased in the vascular bed with abolished tone, vascular sections showed a passive behaviour, the resistances quickly returning to control values after a transient overshoot. In experiments with preserved vascular tone, such behaviour was noted in the venous and to some extent also in the proximal arterial sections, whereas the overall and microvascular resistance vessels exhibited clearcut active dilator responses in terms of significantly decreased resistance compared to the preceding and to the control values. Note that these dilator responses, reaching their maxima soon after the completed pressure decline, were much more pronounced with high than low rate pressure decrease. Few minutes later all resistances had returned to their initial control levels.

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5 Effects of dynamic and static stretch and shortening on mechanical and extracellularly recorded neural activity in the isolated rat portal vein. Lengthening from 6.0 to 8.5 mm at rate of 5 mm/min evoked marked excitatory effects, followed by an afterdischarge (see calculated data for active force and α -act time). At constant increased length, activity stabilized at levels only somewhat above control at initial length. Phasic shortening at the rate of -5 mm/min caused complete abolition of activity. Left panel shows high paper speed recordings during single burst of activity. (Reproduced from Johansson and Mellander *Circulator Res.* 36: 76-83 1975, by permission.)

the stretch and shortening on mechanical and extracellularly recorded electrical activity in the portal vein. Lengthening by 40% from 6.0 to 8.5 mm at a constant rate ($dL/dt = 5$ mm/min, $1/13^{\text{th}}$ of the muscle length/s) evoked marked excitatory effects during the period of dynamic stretch, followed by an afterdischarge (see calculated data for active force and α -act time). During the period of static stretch at the constant increased length, activity stabilized at a level only somewhat above the control value at initial length. Phasic shortening at the rate of -5 mm/min caused complete abolition of electrical and, hence, mechanical activity but control activity reappeared when the muscle had reached its initial control length. Left panel shows high paper speed recordings of a single spontaneous contraction in the control period, the corresponding burst of spikes, and the spike-counter output. The study further showed that the magnitudes of the electrical and mechanical responses to dynamic stretch were distinctly graded in relation to the rate of stretch over a wide range, both for the positive and negative values of dL/dt (Johansson and Mellander 1975).

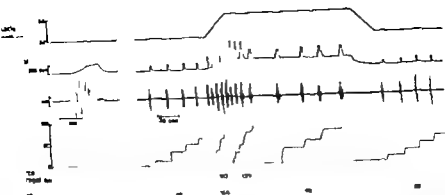
These findings provided direct support not only for the myogenic hypothesis but also for the presence of a "rate-sensitive" as well as a "static" component in the myogenic control, with the former being shown to be the much more effective one. From a subsequent *in vitro* analysis (Sigurdsson *et al* 1977), it appeared that the rate-sensitive myogenic response was more closely correlated to the change in passive force (dP/dt) than to dL/dt . It was also demonstrated that the rate-dependent myogenic response was equally pronounced when the shortening could occur as under conditions when contraction manifested itself only as active force ("isotonic" and "isometric" contractions). Both these observations might be important when considering the *in vivo* situation in view of the facts that passive tension effect was not a prominent feature in the microvessels during transmural pressure increase (see below) and, further, that smooth muscle contraction in resistance vessels as a whole implies active shortening. There is every reason to believe that excitatory and inhibitory

mechanisms did not seem to be involved these vascular reactions most likely can be considered to be myogenic in nature.

However metabolic influences possibly could have attenuated the myogenic responses, once they were developed, as blood flow decreased below the control level during the transmural pressure increase and increased above the control level during the pressure decrease (e.g. Fig. 1). Such an influence thus might have contributed to the rapid decline of the initial myogenic constrictor response to the transmural pressure increase (Fig. 1-4). These flow effects were relatively moderate, however and the metabolic influence must have been quite small in view of a recent finding of Johnson *et al* (1976) that even complete flow cessation by arterial occlusion did not cause any significant metabolic vasodilation in skeletal muscle unless the duration of the occlusion period exceeded 30 s.

The myogenic responses described in this study (e.g. Fig. 4) occurred mainly in the "microvessels" delineated by the sites along the vascular tree from which small artery pressure and small vein pressure, via collaterals, were monitored. The dimensions of these microvessels on the arterial side can be roughly deduced from data for simultaneous pressure and diameter measurements in the muscle microvessels reported by Fronck and Zweifelach (1975). The average value for small artery pressure in the control period of 63 ± 15 (S.D.) mmHg in our material thus implies that it was monitored from arterial vessels in the order of 15-20 μ m. It is known from the mentioned study and others that arterial microvessels of this size and smaller exhibit the greatest responsiveness to several stimuli and the present observations indicate that myogenic reactivity is also mainly confined to these vessels. It cannot be excluded however that the minute venous vessels to some extent contributed to the observed myogenic microvascular effects. It should be noted that the microvascular responses were strong enough to clearly affect total vascular resistance despite the fact that the large veins behaved passively.

Our interpretation of the present results in terms of a "rate-sensitive" and a "static" component in the microvascular myogenic control is mainly based on the finding of a clear cut two-component effector response, the initial one showing distinct quantitative correlation to the rate at which the transmural pressure stimulus was applied, and being temporally separated from the steady state component evoked during the period of constant increased or decreased pressure. The magnitude of the latter component seemed to be pressure amplitude dependent. Some alternative explanations were excluded by special test expts. (see Results). The presence of rate-sensitivity in the myogenic control has previously been suggested from results obtained by other *in vivo* approaches mentioned in the Introduction (Basar and Weiss 1969; Mellander and Arvidsson 1974). Strong arguments for our proposed interpretation can be developed from recent knowledge of vascular smooth muscle physiology. In this respect an analogous *in vitro* study by Johansson and Mellander (1975), in which electrical and mechanical myogenic responses in the rat portal vein to passive stretch were examined in detail, deserves special attention and will be briefly reviewed. The smooth muscle of the portal vein is of the single-unit propagating type and such behaviour is considered to be a dominant characteristic also of the smooth muscle in precapillary microvessels (Mellander and Johansson 1968; Somlyo and Somlyo 1968; Bevan and Ljung 1974). Fig. 5 is a reproduction of one of the mentioned *in vitro* expts. It shows (right panel) the effects of "dynamic" and "static"



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These findings provided direct support not only for the myogenic hypothesis but also for the existence of a "rate-sensitive" as well as a "static" component in the myogenic control, with the former being shown to be the much more effective one. From a subsequent analysis (Sigurdsson *et al.* 1977), it appeared that the rate-sensitive myogenic response was more closely correlated to the change in passive force (dP/dt) than to dL/dt . It was also observed that the rate-dependent myogenic response was equally pronounced when shortening could occur as under conditions when contraction manifested itself as active force (i.e. "isotonic" and "isometric" contractions). Both these observations are important when considering the *in vivo* situation in view of the facts that passive tension effect was not a prominent feature in the microvessels during transmural pressure changes (see below) and, further, that smooth muscle contraction in resistance vessels implies active shortening. There is every reason to believe that excitatory and inhibitory

contractile responses of vascular smooth muscle *in vitro* have a corollary in the *in vivo* situation in terms of vasoconstriction and vasodilation, respectively.

In view of these considerations and of the conspicuous similarity between the pattern of resistance response illustrated in Fig. 4 and the pattern of vascular smooth muscle contractile response depicted in Fig. 5 it seems most likely that the results of the present study can be interpreted in terms of a "rate-sensitive" as well as a "static" component in the myogenic microvascular control *in vivo*.

It follows that the magnitude of the rate-dependent vascular constrictor response to the transmural pressure increase in the present material (Fig. 4) can be roughly estimated by subtracting the steady state resistance response from the initial peak resistance response. Such deductions suggest that the rate-dependent microvascular constrictor response was roughly eight times greater at the high (2.7 mmHg/s) than the low (0.33 mmHg/s) rate of the transmural pressure increase (cf. solid and dashed curves in Fig. 4). The corresponding difference with regard to the overall resistance vessels in the region was in the order of four to fivefold. At the high rate (solid curve in Fig. 4), the rate-dependent "dynamic" microvascular response was more than ten times greater than the "static" response. Note also the promptness of the dynamic responses at both rates, being triggered almost immediately upon the transmural pressure change and fully developed within some 20 s (Fig. 1, 3 and 4).

As mentioned in the Introduction, myogenic activity in vascular smooth muscle is considered to be controlled by a "pacemaker" mechanism which in response to some stimulus related to raised transmural pressure, causes increased impulse discharge propagated to the neighbouring muscle effector cells and, hence, vasoconstriction. The *in vitro* expts. suggested that the sensor element is some kind of a mechano-receptor (Johansson and Mellander 1975, Sigurdsson *et al.* 1976, 1977) responsive to "static" as well as "dynamic" stimuli (ΔL and dL/dt , or ΔP and dP/dt respectively).

The situation *in vivo* is complicated for instance by the fact that the myogenic constrictor response to a given stimulus results in decrease of vessel calibre below the control size (as confirmed by the present results, Fig. 4) which *a priori* would seem to abolish the stimulus, at least if the receptor is responding to increased length. In fact, Fig. 4 indicates that the initial passive distension of the microvessels upon the transmural pressure increase (as judged from the observed decrease in resistance) was so small and shortlasting that it possibly could not have been responsible for the much more prolonged "dynamic" and "static" constrictor responses. If, as suggested by Johnson and Wayland (1967) and Johnson (1974), the sensor element is coupled in series with the contractile elements, receptor elongation might still be maintained during myogenic vessel constriction. An alternative explanation was proposed by Folkow (1964) implying static elongation beyond control length of the sensor element during the period of relaxation of the smooth muscle between repetitive rhythmic contractions. It appears that the present concept of a rate-sensitive sensor can aid in the understanding of maintained receptor stimulation during constriction. If the "static" myogenic constrictor response evoked during the plateau of a pressure increase fails to maintain vascular tone at the new increased level the vessels would again be transiently distended which, in turn, would create the stimulus for a repeated "dynamic" constrictor response. Note in this context that the "dynamic" stimulus (e.g. dL/dt) in contrast to the

tone, is largely independent of absolute sensor length. It is quite possible then that sensor tone during increased transmural pressure will oscillate around an increased level as a result mainly of repetitive dynamic sensor stimulation. Similar events might occur during pressure decrease (possibly explaining the oscillations in Fig. 1). The damping (perhaps specially pronounced at high pressure) and the asynchrony of such cyclic variations in tone are, however, factors which could contribute to the maintenance of an overall relatively stable level of blood flow in the tissue, as usually observed in the steady state in the present experiments.

It might be argued in this context that the rapid decline of the initial constrictor response to transmural pressure elevation (Fig. 4) is just a reflection of an eliminated "static" receptor tone upon vessel constriction and that no "dynamic" stimulus need be invoked. This possibility seems to be refuted by several of the arguments given above, for instance the finding that the initial response was distinctly correlated to the rate of the transmural pressure increase and that the dynamic response *in vitro* during active muscle shortening is equal to that during isometric force development (Shigardsson *et al.* 1976, 1977).

The functional significance of the suggested rate-sensitive component in the myogenic control might be manifold. In general it would seem that, like any servo-mechanism, the myogenic control system would function more properly with receptors sensitive not only to tone deformation but also to the rate of deformation. In a linear system, such a servo-scheme would be much refined by the thereby increased order of the transfer function. An appropriate characteristics of the transfer function (e.g. Meisa and Schultz 1969) the system would show damped oscillatory behaviour. Perhaps this could be an additional explanation of the oscillations, especially in the microvessels, noted upon the transmural pressure decline in Fig. 1. For rapid servocontrol, as implicit in myogenic flow autoregulation, a rate-sensitive receptor mechanism might well be required. It would imply that the faster the transmural pressure increases, the more effective and prompt is the active constriction that counteracts the expected passive vessel distension. Energy expenditure would be much greater if effective constriction were delayed more or less to the moment full passive distension already has developed, especially in view of the effect of Laplace's law which imposes an extra load due to the then much increased passive wall tension.

The model of study used here with an equal increase of transmural pressure across the whole vascular bed resembles the situation in dependent regions when one shifts from supine to erect body posture. The initial load on the dependent vascular bed caused by kinetic energy exceeds several times the later steady state hydrostatic load and the overshoot in microvascular resistance above control noted in the present experiments (Fig. 4) might then be needed to protect the vessels against damage and to cause subsequent proper flow autoregulation. The concept of a dynamic component in the myogenic control can also explain how prompt myogenic vasoconstrictions and vasodilations are accomplished during the transient period of an arterial blood pressure change to compensate quickly for the altered arterio-venous pressure difference and how such responses are graded in relation to the rate of the pressure change.

It has sometimes been said that a positive feedback mechanism is inherent in the myogenic theory (the pressure rise evokes constriction and an increase in resistance which, in turn,

causes an additional pressure rise, etc.) The *in vitro* studies (Johansson and Mellander 1976 Sigurdsson *et al* 1976) strongly indicated that any extreme effects of such a mechanism would not ensue, since there was an upper biological limit of the dynamic response at high rates of stretch. Furthermore, the chemical metabolic control system acts as a brake on the myogenic control system, since intense vasoconstriction is counteracted by the attendant local accumulation of vasodilator metabolites (Berne 1964). The dynamic component in myogenic control might also, as mentioned, favour the development of a damped oscillatory response of the vascular smooth muscle when transmural pressure is changed with consequent alternating periods of excitation and inhibition caused by alternation between repetitive positive and negative rate-dependent stimuli. This implies an inherent negative feedback component in the myogenic control which efficiently might hinder extreme myogenic effects and reactions.

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causes an additional pressure rise, etc.) The *in vitro* studies (Johansson and Mellander 1975 Sigurdsson *et al* 1976) strongly indicated that any extreme effects of such a mechanism will not ensue since there was an upper biological limit of the dynamic response at high rates of stretch. Furthermore the chemical/metabolic control system acts as a brake on the myogenic control system, since intense vasoconstriction is counteracted by the attendant local accumulation of vasodilator metabolites (Berne 1964). The dynamic component in myogenic control might also, as mentioned, favour the development of a damped oscillatory response of the vascular smooth muscle when transmural pressure is changed with consequent alternating periods of excitation and inhibition caused by alternation between repetitive positive and negative rate-dependent stimuli. This implies an inherent negative feedback component in the myogenic control which efficiently might hinder extreme myogenic effector reactions.

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Characteristics of Aortic Baroreceptor C Fibres in the Rabbit

By

PETER THOREN and JOHN V JONES

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Abstract

THOREN P and J V JONES. *Characteristics of aortic baroreceptor C fibres in the rabbit*
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The characteristics of 40 C-fibres arising from the aortic arches of 15 normotensive rabbits have been investigated. The conduction velocity of the fibres varied between 0.5-1.8 m/s (0.91 ± 0.05 mean \pm S.E.). The activation threshold for all the fibres lay between 70-140 mmHg. 12 medullated fibres from the same animals had thresholds between 35 and 90 mmHg. After correction for the time delay, conduction of the firing of the C-fibres occurs in early systole. Pressure response curves were constructed for 10 medullated and 11 C-fibres. The mean activity at 100 mmHg was, for the C-fibres 3.0 Hz and for the medullated fibres 34 Hz. At 130 mmHg the activity of the C-fibres was 13 Hz and in the medullated fibres 68 Hz. Thus C-fibres from the aortic baroreceptor regions are activated at higher pressures than the medullated fibres and have lower discharge frequencies. Noradrenaline did not influence the pressure response curves of the C-fibres. It is concluded that the arterial baroreceptor C-fibres may exert a tonic influence on the vasomotor centre at normal pressures but are likely to be of greater importance when the arterial pressure rises acutely.

Aortic baroreceptors with medullated afferents have been studied extensively in many species e.g. dogs, rabbits and rats (Kirchheim 1976). However the aortic nerve also contains non-medullated fibres. Electrical stimulation of these non-medullated afferents in the aortic and also in the carotid sinus nerves can evoke powerful depressor reflexes (Douglas and Ritchie 1956, Douglas, Ritchie and Schaumann 1956) with different efferent patterns from the medullated fibres (Kardon, Peterson and Bishop 1975). Activity in non-medullated baroreceptor afferents was first recorded by Fidone and Sato (1969). However Landgren (1951) described baroreceptor afferents in the sinus nerve with a low signal to noise ratio which were probably C-fibres. Coleridge *et al.* (1973) also studied aortic C-fibres. These C-fibres in the dog and cat had very high thresholds. Recently Thoren, Saum and Brown 1976 have studied the characteristics of aortic C-fibres using an *in vitro* aortic arch preparation in rats. The C-fibre receptors in the rat had higher thresholds than the medullated receptors. Furthermore, they had only a sparse discharge at normal pressure but could be markedly activated when the pressure was elevated.

On leave from the University Department of Medical Cardiology Royal Infirmary Glasgow Scotland

This study was undertaken to examine the characteristics of C-fibres arising from the aortic arch of the rabbit. In comparison with the previous work in the rat, this study was done on an *in vivo* preparation in order to keep the sympathetic efferent fibres to the aortic arch intact and to avoid artificial perfusion media. In addition the characteristics of C-fibre receptors were compared with those of medullated aortic baroreceptors from the same animal.

Methods

Experiments were performed on rabbits (1.5–2.5 kg b.w.). The animals were anaesthetized with sodium methohexital (Mehobexal, 30 mg/kg b.w.) after premedication with diazepam (Valium 5 mg). Additional doses of 5–10 mg/kg Nembutal were given at intervals as required. A tracheal cannula was inserted and the animal put on positive pressure ventilation with 100% oxygen at an end-tidal pressure of 10 cm H₂O. Muscle temperatures were prevented by means of gallamine triethiodide (Flaxedil, 10 mg/kg repeated injections). Rectal temperature was measured and maintained at 37–38°C by means of a heating lamp. The PO_2 , PCO_2 and pH were measured at intervals and PCO_2 and pH were maintained in the normal range (38–40 mmHg and 7.30–7.45 respectively) by adjusting the tidal volume and by adding small amounts of NaHCO₃ (1 meq/l) as required.

On the day, the aortic nerves, the sympathetic trunks and the carotid arteries were dissected free. The thorax was opened by bilateral transverse incisions and incisions were placed around the descending aorta, close to the diaphragm, and around the inferior vena cava. Through a slit in the pericardium the aortic nerve was exposed thus allowing it to be briefly clamped with forceps for localization of receptors. The arterial blood pressure was measured with a Statham transducer (P23 DC) connected to a catheter inserted at the arch of the aorta via one femoral artery. The arterial pressure was recorded on a polygraph (model 7 D) and on an ultraviolet (UV) light recorder (ABEM 5651). One femoral vein

was cannulated. Bipolar electrocardiograms (ECG) were recorded with one electrode inserted in the left side of the chest and the other electrode placed in the oesophagus behind the heart. The ECG was displayed on both the oscilloscope (Tektronix 502) and on the UV-recorder.

Isolation of aortic traffic

The left aortic nerve (in 2 rabbits the right aortic nerve) was placed on small plates. A pool of saline was made in the surrounding muscle and this pool was filled with mineral oil. The nerve sheath around the aortic nerve was removed by means of sharp forceps under binocular dissection microscope and thin filaments of the filaments were placed on bipolar chloridured silver electrode and connected to a bipolar amplifier. The output was displayed both on the oscilloscope and on the UV-recorder. The amplifier was connected to a loudspeaker and to a stimulator (time constant 0.4 s) equipped with a discriminator which could count either all spikes exceeding a preset level or only spikes with amplitudes between two determined levels. The output of the stimulator was recorded on the Grass polygraph. The activity of receptors was calculated from the spike counter recordings but numerous spot checks of the actual recorder tracings were made in each experiment to ascertain the accuracy of the spike counter readings.

The conduction velocities of the fibres could be established from the time delay between an electrical stimulus (Grass S4) applied to the aortic nerve, close to its entrance into the thorax, and from the distance between the stimulating and recording electrodes. Whenever possible the total conduction time was measured by applying the stimulating electrode to the area on the aortic arch where the receptor could be stimulated mechanically.

Microstimulation procedures

Activity in all filaments was observed during electrical stimulation of the aortic nerve close to the thorax. Filaments showing evidence of aortic C-fibre activity were dissected further until single or few were remaining active. All filaments showing evidence of C-fibre traffic were then tested by short-circuiting the descending aorta.

The localization of the receptors to the aortic arch was established by the response to occlusion of the descending aorta and the lack of response to brief occlusion of the ascending aorta. In many cases the exact anatomical location of the receptor was obtained by gently exploring the aortic arch with a fine blunt probe. Receptor activity in single or few fibre preparations was observed with alterations in aortic pressure either by means of graded caval vein occlusion or by means of graded occlusion of the descending aorta.

The blood pressure/receptor response curve was also determined during intravenous infusion of noradrenaline.

Results

The study is based on recordings from 40 aortic C fibres and 32 aortic medullated fibres in 15 normotensive rabbits. The awake mean arterial blood pressure (\pm S.E.) measured directly i.a. in the ear artery (21 g needle) was 83 ± 8 mmHg. The conduction velocity for the C fibres varied from 0.5–1.8 m/s (0.91 ± 0.05 mean \pm S.E.).

Location of the receptors

15 of the 40 receptors were located in the aortic arch by gentle probing with fine forceps. These 15 receptors were located throughout the entire aortic arch. The other 25 receptors were localised to the aortic arch by the augmented receptor activity during occlusion of the descending aorta and the lack of receptor response or decrease in activity during occlusion of the ascending aorta. It was also evident that the aortic nerve in the rabbit contains fibres from receptors which responded to occlusion of the ascending aorta meaning that these receptors were probably located in the heart. This was confirmed in many cases by mechanical probing of the heart. These cardiac C-fibres were not subjected to further study. Furthermore a few C-fibres with a sparse discharge related to the respiratory cycle were also found.

Threshold for activation

The threshold mean arterial blood pressure for activation of each of the 40 receptors with non-medullated afferents is shown in Fig. 1. As a comparison the threshold values for all 32 medullated receptors are also shown. Only 10 of the 40 C-fibre receptors had threshold levels lower than the mean pressure in the unanaesthetized animal (83 mmHg) while almost all the medullated fibres had threshold values lower than the mean ambient blood pressure. In general there is little overlapping in the thresholds of the medullated and non-medullated fibres.

The receptor response to a graded change in arterial pressure

The receptor response to graded changes in aortic arch pressure was tested in 10 medullated and 18 C-fibres.

Fig. 2 shows an example of the activity in a single aortic C-fibre during a graded caval and aortic occlusion. The receptor has a spontaneous discharge of about 1 imp/heart beat (A) at a mean arterial blood pressure (MABP) of 100 mmHg. During the caval occlusion the receptor stops firing at a mean pressure of 95 mmHg. During graded occlusion of the descending aorta the activity increases in parallel with the rise in arterial pressure up to a maximum value of more than 20 Hz. The firing initially displays a clear cardiac rhythmicity (B, C) but becomes continuous (D) at high pressure.

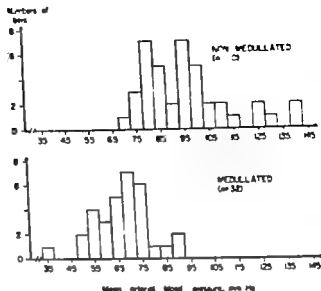


Fig. 1 The threshold blood pressure for activation of 46 non-medullated and 32 medullated fibres.

Fig. 3 shows the mean blood pressure/receptor response curve for 18 C-fibres and 10 medullated fibres during graded inferior vena caval and aortic occlusion. Many of the receptors with high thresholds could not be tested by graded occlusion of the descending aorta because the highest pressure then obtained during occlusion was just barely enough to activate these high threshold receptors. Furthermore it is highly probable that there exist some C-fibres with thresholds above the maximum blood pressure attained since many filaments contained C-fibres which could not be activated by aortic occlusion. These non-responsive fibres were not arising from chemoreceptors as they were not activated by apnoea. The pressure response curve for the C-fibre discharge in Fig. 3 is thus not a correct reflection of the activity in the whole spectrum of aortic C-fibres because many high threshold receptors could not be included.

The firing pattern in the cardiac cycle

The firing pattern in relation to the cardiac cycle was examined for 9 receptors in which the total conduction time (50–130 m/s) from the receptor site to the recording electrode was known. By taking the time into account the corrected position of the receptor activation in the cardiac cycle could be determined. Fig. 4 is an example of this. This filament contains 1 C-fibre with prominent upward deflection and 2 medullated fibres with more prominent downward deflections. The total conduction time (90 m/s) from the receptor site in the apex of the aorta was measured in C and by taking this delay into account the timing of the receptor activation in relation to the cardiac cycle could be established as indicated by the marks in A and B. The C-fibre is recorded as firing in late systole or in diastole but the receptor activation actually occurs in early systole in phase with the activation of the medullated fibres in the filament.

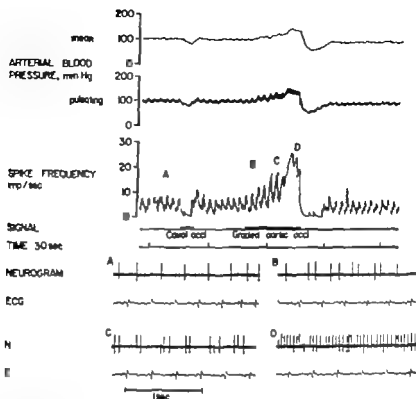


Fig. 2 Effects of graded occlusion of the inferior caval vein and the descending aorta on aortic blood pressure and spike frequency in a single aortic C-fibre. The letters in the spike frequency recordings correspond to the neurogram below.

The effect of noradrenaline on the receptor discharge

The blood pressure/receptor response curve for 18 C-fibre receptors was compared during mechanical obstruction of the descending aorta and during injection of noradrenaline (NA) as shown in Fig. 5. 15 of the receptors were studied when the NA was given in repeated bolus injections and 3 receptors during continuous infusion of NA ($9 \mu\text{g}/\text{min}$) for more than 5 min. The blood pressure rise consequent upon the infusion of NA was prevented by partial caval occlusion. Towards the end of the infusion the pressure response curves were determined by gradual release of this occlusion which resulted in a blood pressure rise. These two ways of administering NA gave similar results and there was no significant difference between the pressure response curves obtained during mechanical occlusion of the descending aorta or during administration of NA. However it is likely that small differences would be missed in this comparison since the pulse pressure with caval occlusion or NA bolus injections was different from that present with aortic occlusion although the mean pressures could be kept the same.

Discussion

The existence of a large population of aortic baroreceptor C fibres (Landgren 1952, Landgren, Neil and Zotterman 1952, Fidone and Sato 1969, Coleridge *et al.* 1973, Thoren, Saum

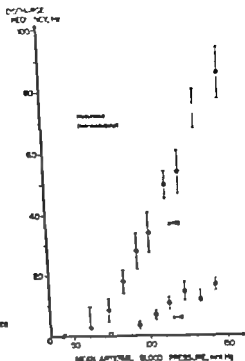


Fig. 3 Mean blood pressure/receptor response curves for 10 C-fibres and 10 medullated fibres.

and Brown 1976) was confirmed in this study. The receptors were distributed throughout the whole aortic arch with their afferent connections in the aortic nerves and with conduction velocities of 0.5–1.8 m/s.

Receptor characteristics

In the control situation the receptors discharged with a low frequency or were silent. At the mean blood pressure (83 mmHg) of this group of animals only 25 per cent of the C-fibre receptors show evidence of activation in contrast to 95 per cent of the medullated fibres. Furthermore at that pressure the mean frequency of 18 tested C-fibres was 1.1 Hz in contrast to 16 Hz in 10 medullated fibres. There was little overlapping in thresholds for the medullated receptors and the C-fibre receptors. The C-fibres were activated in systole just as the medullated fibres and noradrenaline infusion did not seem to alter their pressure response curves significantly. These data in an *in vivo* rabbit preparation agree with an earlier study by Thoren, Samn and Brown (1976) on an *in vitro* rat aortic arch preparation. The C-fibres arising from the rat aortic arch thus also seem to have higher thresholds and lower discharge frequencies than the medullated fibres. In the aortic arch preparation of the rat 2 different types of aortic C-fibres were described. One group with an irregular rather low frequency discharge and one group with a regular and more high frequency discharge. A similar analysis of the receptor discharge cannot be performed in the present study because it is not possible to alter the pressure in a square wave fashion in this *in vivo* preparation. However even in the present preparation there is marked variation in the receptor response to aortic

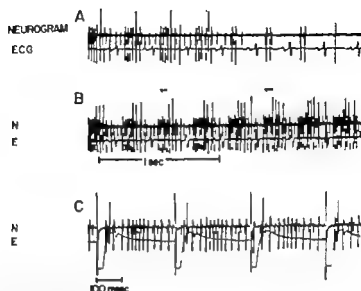


Fig. 4. Firing pattern in relation to the cardiac cycle for 11 C-fibre (prominent upward deflection) and medullated fibres (downward deflections). A. During carotid occlusion. B. During descending aortic occlusion. C. Total conduction time obtained by electrical stimulation over the receptor area. Sign indicates corrected position of receptor activation in cardiac cycle after total conduction time as measured in B taken into account.

occlusion. Some receptors responded at moderate pressures with a high frequency and relatively regular discharge (up to 42 Hz) while other receptors responded with only a low frequency sporadic discharge even at high pressures. However, whether the same difference between regular and irregular receptors as seen in the rat also exists in the rabbit cannot be established with the present preparation.

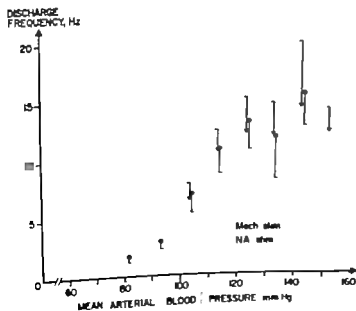


Fig. 5. Blood pressure receptor response curves for 11 C-fibres obtained when blood pressure was varied by means of mechanical occlusion of the descending aorta (circles) and by means of injections or infusion of noradrenaline (triangles).

In present study further supports the classical study by Landgren (1952) where he labelled carotid sinus baroreceptor fibres with low signal to noise ratio which presumably are C-fibres. These receptors had a higher threshold and a lower discharge frequency than the medullated fibres arising from the carotid sinus. This study does not agree with the study on aortic C-fibres in the dog by Coleridge *et al.* (1973). They claimed that the aortic C-fibres had very high and unphysiological thresholds. The difference in our results might of course be due to species differences. In the studies of Coleridge *et al.* the fibres were evidently dissected mainly from the main vagus stem and not from the aortic nerve. It is at least a possibility that the aortic baroreceptors travelling in the vagus might have somewhat different characteristics. Furthermore the aortic fibres in their study were selected according to their responses to drugs, while in the present study the receptors pressure responses were determined.

Many filaments were examined containing C-fibres that could not be activated even by maximal pressure increases in the aortic arch. Other C-fibres were also studied which had such high thresholds that the pressure could not be raised far enough to obtain other than threshold values for these receptors. Therefore the pressure response curves presented here represent those C-fibres with lower thresholds and are, therefore, not representative of the whole group.

Location of receptor activation

Aortic C-fibres respond to increased pressure in the aortic arch and they are activated during the systolic pressure wave as are aortic medullated receptors. It is therefore likely that these receptors, as well as the medullated fibres, respond to stretch of the aortic wall. Furthermore there is no data indicating that contraction of the smooth muscle elements in the aortic wall with NA might influence the pressure response curves and thus that the aortic C-fibres are coupled in series with the smooth muscle elements in the wall. This is in contrast to earlier data by Landgren, Neil and Zotterman (1952) and Thoren, Saum and Neil (1976). However in these expts. much stronger solutions of NA were used. Thus, Landgren *et al.* (1952) applied a stock solution of NA (1 mg/ml) on the sinus wall while Thoren *et al.* (1976) perfused the rat aortic arch with a solution containing 1 µg/ml which is a considerably stronger concentration of NA than was used in the present study. It is possible that NA in very high doses may affect the pressure response curves but within the limitations of this study the data does not indicate that such an effect is of importance at physiological concentrations of NA.

Baroreceptor effect and physiological role of aortic baroreceptor C-fibres

Baroreceptor effects arising from aortic baroreceptor C-fibre activation during differentiated electrical stimulation of the aortic nerve in the cat and the rabbit was demonstrated by Douglas and Ritchie (1956) and Douglas *et al.* (1956) who showed that the aortic C-fibres induced a vagal depressor reflexes, at low stimulation frequencies. These reflexes were different from those arising from aortic baroreceptors with medullated fibres during electrical stimulation. The C-fibres had their main effect on the vagal outflow to the heart with only little effect on the sympathetic outflow (Kardon, Peterson and Bishop 1975).

The discharge in aortic C-fibre baroreceptors normally is sparse at the ambient pressures of these normotensive rabbits (range 70–90 mmHg). Thus the medullated fibres, because of their lower thresholds, may be the more important fibre group under normal conditions or when the blood pressure is reduced such as occurs with haemorrhage. However during acute increases in blood pressure such as occurs in many everyday situations, C-fibre traffic will increase and have a greater inhibitory effect on the vasomotor centre. Possibly they might even surpass the medullated fibres in importance under these circumstances.

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Alpha-Receptor Subsensitivity of Isolated Atria from Rats Following Physical Training or Repeated ACTH Injections

By

ANNE SULTOVUORI, RAUNO TIIRI and MIKKO N. E. HARRI

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Abstract

SULTOVUORI, A., R. TIIRI and M. N. E. HARRI. *Alpha-receptor subsensitivity of isolated atria from rats following physical training or repeated ACTH-injections*. Acta physiol. scand. 1977 99 457-461.

Decreased chronotropic and inotropic sensitivity of isolated atria to phenylephrine but not to isoprenaline was found in rats following repeated physical training by swimming or repeated ACTH-injections. These changes were induced simultaneously with subsensitivity of animals to these treatments. It is concluded, that these effects are mediated by increased adrenergic activity which results in the subsensitivity of alpha-receptors.

Lowered sensitivity of cardiac α -receptors is induced in rats by cold-exposure (Harri *et al.* 1974) and by repeated injections of phenylephrine or isoprenaline (Tiiri *et al.* 1976), as indicated by lowered atrial chronotropic response to an α -adrenergic drug phenylephrine. On the contrary an increased sensitivity of cardiac α -receptors is found to be induced in dog and/or humans by several conditions associated with low physiological activity of the animal. Such conditions are the inactivity of skeletal muscles (Szenteiványi *et al.* 1970 a), propylthiouracyl treatment (Nakashima *et al.* 1971), hypothyroidism (Kucos *et al.* 1974), increased vagal influences on heart (Szenteiványi *et al.* 1970 b) and low experimental temperature of the heart (Kucos and Szenteiványi 1968, Buckley and Jordan 1970, Harri 1973). These effects are usually associated with lowered β -receptor sensitivity shown in inotropic and/or metabolic activity of the heart. Supersensitivity to the chronotropic effects of noradrenaline has been shown to appear in the dog heart in 1 to 3 days of treatment with morphine (Fleming and Trendelenburg 1961, Westfall and Fleming 1968). This supersensitivity seemed to be nonspecific because it occurred also using calcium.

To find out a possible common physiological condition, we further studied the effect of physical stress conditions and repeated ACTH-injections on the adrenergic response of isolated atria.

Material and methods

A total of 112 adult male Sprague-Dawley rats, 200–300 g in weight, were used in these experiments. 48 of them were daily exercised by swimming for 20 min in water at 30°C for 6 or 20 days. A weight of 7 g was attached at the base of the tail. An equal number of intact rats served as the control group. A group of 14 animals received once daily s.c. injection of adrenocorticotrophic hormone (3 IU/kg) for a week. A equal group of rats received injections of NaCl-solution.

The cumulative concentration-response curves for the response to isoprenaline (ISO) and phenylephrine (PHE) were determined using isolated atria at 37°C in Tyrode's solution. The number of animals in each group is presented in the graphs.

To measure the chronotropic response to PHE and ISO the animals were killed by decapitation. The atria were dissected from the hearts and suspended horizontally with tension of 600 mg in 30 ml of Tyrode's solution gassed with 95% O_2 –5% CO_2 . The rate of spontaneous contractions was recorded on Mingograph 4 jet recorder by means of a suction electrode as described earlier (Hirri *et al.* 1974). For measuring the inotropic responses to PHE and ISO the cranial tip of the left atrium was suspended in Tyrode's solution by means of the pin with platinum electrodes on them. The caudal end was fixed in position with hook connected to the force transducer on the Grass polygraph recorder. The contractions of the atrium were driven electrically from the stimulator at a steady rate of 120 beats per min. Square wave pulses with double threshold intensity (about 2.5 V) and a duration of 3 ms were used. The resting tension applied was 600 mg, otherwise the conditions were the same as in the measurement of chronotropic response. A single concentration-response curve was determined with each trial preparation. The following drugs were used: isoprenaline hydrochloride (Isuprel, Winthrop), phenylephrine hydrochloride (Neo-synephrine, Winthrop) and adrenocorticotrophic hormone (Isactid, Ferring).

Results

The graphs in Fig. 1 show that after 6 and 20 days of physical training by swimming, the positive chronotropic sensitivity to PHE was lowered. This can be seen in the concentration-response curves shifted to the right. Because the maximum responses were strongly reduced ($p < 0.01$), the EC_{50} -values did not differ significantly in any of the treated groups. The basic contraction frequency of rat atria was not changed after the swimming exercise as compared to the controls being 233 ± 6 and 226 ± 7 beats per min respectively.

The results of the inotropic responses to PHE and ISO are presented in Fig. 2. The graphs show that physical training by daily swimming for 6 days reduced the sensitivity to PHE but did not change the response to ISO. The results are thus similar to those in chronotropic response. The basic contractile force was slightly but not significantly greater in rats after training by swimming than in controls (176 ± 16 mg and 147 ± 14 mg, respectively, $p = 0.20$).

The results in Fig. 3 indicate the effects of repeated ACTH injections for 7 days on adrenergic atrial response. The chronotropic sensitivity to PHE was lowered after this treatment. The maximum response to PHE was reduced to about 50% after ACTH-injections as compared to the control values and it was statistically significant ($p = 0.05$). The responses to ISO were the same in the control and experimental group. ACTH treatment did not cause any changes in the basic contraction frequency.

Discussion

The results of the present study are similar to those found in isolated atria from cold-exposed rats (Harri *et al.* 1974) and from rats injected chronically with PHE or ISO (Tirri *et al.* 1976). In all these studies the lowered chronotropic sensitivity to the α -adrenergic drug, PHE, was observed in isolated atria, but there was no change in response to the β -adrenergic

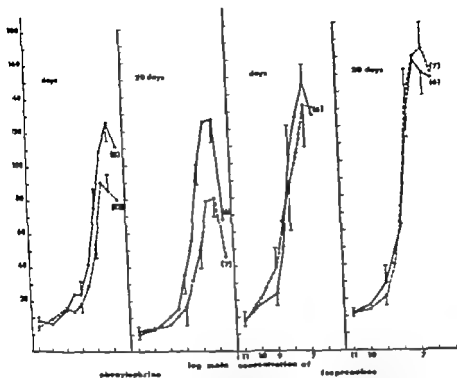


Fig. 1. Log concentration-response curves for the chronotropic responses to phenylephrine (A) and noradrenaline (B) *in situ* from control rats (continuous lines) and rats trained by daily swimming for 6 or 20 days (broken lines). The number of animals in each group is in brackets and vertical bars indicate \pm S.E.

ing ISO. It could be assumed that the enhanced adrenergic activity in all these experimental conditions was responsible for the subsensitivity of α -receptors observed (Harri *et al.* 1974, Törn *et al.* 1976). The higher level of noradrenaline, as produced by cold, repeated injections of adrenergic drugs or physical training, could thus account for this lowered sensitivity of α -receptors. The subsensitivity induced by ACTH-injections may be a compensatory action of the stimulating effect of corticosteroids on the adrenergic action in heart muscle as shown *in vitro* by Kaumann (1972). On the other hand, the decreased sympathetic activity in rats resulting from decentralization or 6-hydroxydopamine treatment causes supersensitivity to noradrenaline but not to ISO (Trendelenburg 1963, Johansson 1973). This may also suggest a role of α -receptors in this supersensitivity. Kunos *et al.* (1973) have suggested that cardiac α - and β -receptors represent allosteric conformations of the same structure. They further suggest (Kunos *et al.* 1974) that changes in the thyroid hormone level result in an interconversion of myocardial α - and β -receptors. Our present and previous results indicate that the subsensitivity of α -receptors was not associated with a simultaneous increase in β -receptor response. This indicated that there was no interconversion of these receptors; thus giving support to Benfey (1975) who has also reported an opposing suggestion to the adrenoreceptor transformation hypothesis.

Material and methods

A total of 112 adult male Sprague-Dawley rats, 700–300 g in weight, were used in these experiments. 48 of them were daily exercised by swimming for 70 min in water at 30°C for 6 or 70 days. A weight of 7 g was attached at the base of the tail. An equal number of intact rats served as the control group. A group of 14 animals received once daily s.c. injections of adrenocorticotrophic hormone (3 IU/kg) for a week. An equal group of rats received injections of NaCl-solution.

The cumulative concentration-response curves for the responses to isoprenaline (ISO) and phenylephrine (PHE) were determined using isolated atria at 37°C in Tyrode's solution. The number of animals in each group is presented in the graphs.

To measure the chronotropic response to PHE and ISO the animals were killed by decapitation. The atria were dissected from the hearts and suspended horizontally with a tension of 600 mg in 30 l Tyrode's solution gassed with 95% O₂–5% CO₂. The rate of spontaneous contractions was recorded on a Mingograph 24 jet recorder by means of a suction electrode as described earlier (Harri *et al.* 1974) measuring the inotropic responses to PHE and ISO the cranial tip of the left atrium was suspended in Tyrode's solution by means of the pins with platinum electrodes on them. The caudal end was free in position with a hook connected to the force transducer on the Grass polygraph recorder. The contractions of the atrium were driven electrically from the stimulator at a steady rate of 170 beats per minute. Square wave pulses with double threshold intensity (about 5 V) and duration of 3 ms were used. The resting tension applied was 600 mg, otherwise the conditions were the same as in the measurement of the chronotropic response. A single concentration-response curve was determined with each atrial preparation. The following drugs were used, isoprenaline hydrochloride (Isuprel® Winthrop), phenylephrine hydrochloride (Neo-synephrine® Winthrop) and adrenocorticotrophic hormone (Isactid® Ferring).

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The graphs in Fig. 1 show that after 6 and 20 days of physical training by swimming, the positive chronotropic sensitivity to PHE was lowered. This can be seen in the concentration-response curves shifted to the right. Because the maximum responses were strongly reduced ($p < 0.01$) the EC₅₀-values did not differ significantly in any of the treated groups. The basic contraction frequency of rat atria was not changed after the swimming exercise as compared to the controls being 233 ± 6 and 226 ± 7 beats per min respectively.

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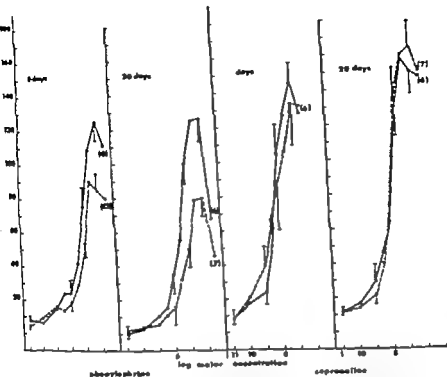


Fig. 1. Log concentration-response curves for the chronotropic responses to phenylephrine (A) and isoproterenol (B) in atria from control rats (continuous lines) and rats trained by daily swimming for 6 or 28 days (broken lines). The number of animals in each group is in brackets and error bars indicate S.E.

drug, ISO. It could be assumed that the enhanced adrenergic activity in all these experimental conditions was responsible for the subsensitivity of α -receptors observed (Harri *et al.* 1974, Tani *et al.* 1976). The higher level of noradrenaline, as produced by cold, repeated injections of adrenergic drugs or physical training, could thus account for this lowered sensitivity of α -receptors. The subsensitivity induced by ACTH-injections may be a compensatory action of the stimulating effect of corticosteroids on the adrenergic action in heart muscle as shown *in vitro* by Kaumann (1972). On the other hand, the decreased sympathetic activity in rats resulting from decentralization or 6-hydroxydopamine treatment causes hypersensitivity to noradrenaline but not to ISO (Trendelenburg 1963, Johansson 1973). This may also suggest a role of α -receptors in this supersensitivity. Kuros *et al.* (1973) have suggested that cardiac α - and β -receptors represent allosteric conformations of the same structure. They further suggest (Kuros *et al.* 1974) that changes in the thyroid hormone level result in an interconversion of myocardial α - and β -receptors. Our present and previous results indicate that the subsensitivity of α -receptors was not associated with a simultaneous increase in β -receptor response. This indicated that there was no interconversion of these receptors, thus giving support to Benley (1975) who has also reported an opposing suggestion to the adrenoreceptor transformation hypothesis.

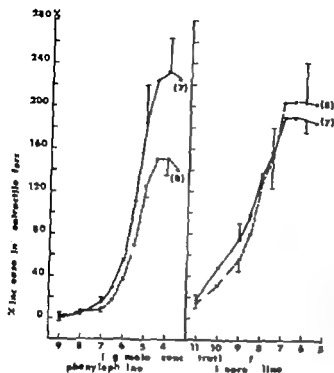


Fig. 2. Log concentration-response curves for the inotropic responses to phenylephrine and isoprenaline in atria from control rats (continuous lines) and rats trained by swimming for 6 days (broken lines). The number of animals is in brackets and vertical bars indicate + or - S.E.

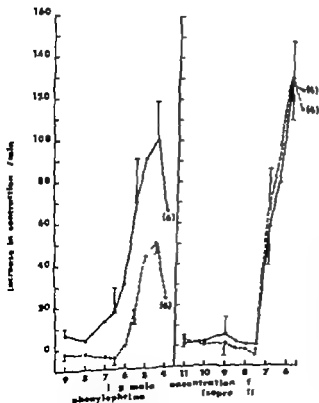


Fig. 3. Log concentration-response curves for the chronotropic responses to phenylephrine and isoprenaline in atria from control rats (continuous lines) and rats injected with ACTH (3 IU/kg) for a week (broken lines). The number of animals is in brackets and vertical bars indicate + or - S.E.

of thyroxine as an inducer of changes in receptor sensitivity cannot be excluded. It is shown that cold-exposure (Heroux 1960) and β -adrenergic stimulation by ISO (et al. 1975) induce thyroid hormone secretion. Thus a change in sympathetic effects changes in thyroxine levels (Melander et al. 1977). On the other hand, the adrenergic action can be induced by an increase of thyroxine hormone level, in hypothyroid or young animals (Swanson 1956, Lagerstedt et al. 1966). Thus, adrenergic sensitivity even when caused by altered thyroxine level, could also be induced by a changed adrenergic action.

It is supported by The Academy of Finland.

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Effect of Hypoxia on Mitochondrial Mass and Cytochrome Concentrations in Rat Heart and Liver during Postnatal Development

By

VUOKKO L. KINNULA and ILARO HASSINEN

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Abstract

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Cytochrome concentrations of rat heart and liver mitochondria were measured postnatally from new-born animals to young adults. Mitochondrial cytochrome aa₃ concentration increased shortly after birth in both tissues, this concentration being in a new-born animal 0.149 ± 0.077 nmol/mg protein in liver and 0.108 ± 0.082 nmol/mg protein in heart. The respective values from a two week old animal were 0.216 ± 0.011 and 0.583 ± 0.100 . Postnatally cytochromes c + c₁ and b₅ increased markedly in the heart, but in the liver of new-born animals the cytochrome content was more close to the adult values. The amount of mitochondrial protein increased after birth, too. In a new-born animal the mitochondrial protein values were 39.7 ± 1.5 mg/g wet weight in liver and 6.6 ± 6.5 mg/g wet weight in heart. In adult animals the respective values were 71.5 ± 4.8 and 80.7 ± 13.1 . The effect of postnatal hypoxia (10% O₂, 4 h and 48 h) on liver and heart mitochondrial cytochrome concentration and protein values of new-born animals were investigated. During hypoxia the amount of mitochondrial protein remained about at the level of a new-born animal. The postnatal increase in the mitochondrial cytochrome concentration, which was smaller in the liver than in heart, was also inhibited by hypoxia.

Key words: Heart, liver, hypoxia, mitochondria, cytochromes.

In mammals, the formation of functional mitochondria is already almost complete during intrauterine life (Hallman 1971). However, in various rat tissues the activities of some mitochondrial enzyme systems, such as cytochrome oxidase, the succinate oxidase system, succinate dehydrogenase and succinate-cytochrome b₅ reductase are low in the fetus and new-born as compared to the adult (Hallman 1971, Lang 1965, Jakovcic *et al.* 1971, Gregson & Williams 1969, Levy and Toury 1970). Also morphological changes in mitochondria occur postnatally. Fetal mitochondria at term are large as compared with those from mature animals (Jakovcic *et al.* 1971). In addition it has been observed that a disproportionate increase in the mitochondrial mass occurs postnatally compared with the overall growth of the organ parenchyma during the same period, at least in the liver (Lang and Herberich 1977).

be synthesis of many components of the mitochondrial respiratory chain is accelerated during the perinatal period (Jakovick *et al* 1971). The concentration of cytochromes increases shortly after birth in different rat tissues (Hallman *et al* 1972, Cherpilinsky and Inguez de Lores Arnaiz 1970, Lisková *et al* 1974). Factors that control these changes are more or less unknown. To our knowledge, only one previous report exists on the effects of oxygen on mitochondrial development postnatally and rather short experimental periods are used (Hallman 1971). The purpose of the present study was to investigate the role of oxygen in regulation of mitochondrial population and cytochrome content after birth and design experimental conditions to allow longer observation periods.

Material and methods

Male rats were used and the age of the animals varied from newborn to 2 week old animals. In addition, female animals (age 90–100 days) of both sexes were used. Newborn rats were separated from their mothers placed in plastic hypoxic chamber in an atmosphere of 10% O₂ in nitrogen at 101 kPa (760 mmHg) levels of 24 h and 48 h. Temperature was maintained at 21°C with relative humidity nearly 100% and the CO₂ concentration as low as possible, soda-lime CO₂ absorbers are incorporated in the circuit. The gas flow was kept about 8 litres/h. In control groups were maintained simultaneously for 24 h and 48 h in normal atmosphere, those with mother and those separated from their mother. The animals were decapitated and all haemogenates were taken from the adults are prepared from pooled tissue of the animals to obtain sufficient amounts of material for analysis. Liver mitochondria are isolated in medium consisting 0.27 M sucrose, 1.0 mM Tris-Cl (pH 7.4). The mitochondria were sedimented at 3200 g by typical Schauder tube (Kawachi 1974). Heart mitochondria were isolated with Bacillus subtilis affinis (obtained from Novo Industri A/S, Denmark) according to Tyler and Geare (1967). The isolation medium was 1 M sucrose, 0.075 M sucrose and 0.05 mM EDTA (pH 7.4). One subsample of the fractions was carried for cytochrome assays were performed using a split beam spectrophotometer with 2 mm bands with cuvettes (Eberle Double Beam 124) essentially according to Hallman *et al.* (1972). The concentration of mitochondrial protein in the tissue was calculated from the cytochrome aa₃ content in the total homogenate and isolated mitochondria (Schellhauer and Klingenberg 1962). Protein was determined by the method of Dy *et al.* (1951).

Results

Average weight of a newborn animal was 5.24 ± 0.4 g. Attempts to induce nursing of the newborn rats by the mother after prolonged adaptation period and parturition in the true atmosphere were not successful. As a result, the newborn had to be deprived of the mother and kept without food. To test the effect of fasting, one group of animals was maintained under normal conditions separated from their mother. During the hypoxic period of 10–30% of the animals died depending on the litter; the respective value of the fasting rats was 0–10%. Cytochrome concentration in the heart is markedly greater than in the liver. In both tissues especially cytochromes aa₃ concentration increased after birth. These results are cited in Fig. 1. Most prominent increase occurred during the first two days after birth. A similar increase was also observed in cytochromes c and b postnatally. Both total and mitochondrial protein concentration increased after birth in both tissues (Table I). In a newborn animal the content of mitochondrial protein in the tissue was 39.7 ± 3.6 mg/g wet weight of liver and 26.6 ± 6.5 mg/g wet weight in the heart. In adult animals the respective values were 71.5 ± 4.8 and 80.7 ± 13.1 .

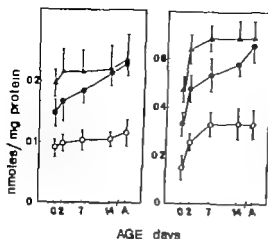


Fig. 1 Mitochondrial cytochrome concentrations in rat heart and liver during postnatal development. Values are means of 6-10 determinations. ●—● cytochrome a_3 , ○—○ cytochrome b and ▲—▲ cytochromes c+c. Left: liver mitochondria. Right: heart mitochondria. The vertical bars represent S.D.

Mitochondrial protein in the tissues in the hypoxic groups remained significantly lower than in the control group or in the starved group (Table I). Concerning the mitochondrial cytochrome content from one- and two-day-old animals, it could be noticed that in both tissues greatest concentrations were obtained in the control group. After the hypoxic period of 24 h the cytochrome content in both tissues was lower than in the fed or fasting control groups. These differences remained qualitatively the same after the 48-hour observation period, the inhibition of the cytochrome induction was largest in the hypoxic groups, but an inhibition of a smaller extent occurred also in the fasting control group (Fig. 2).

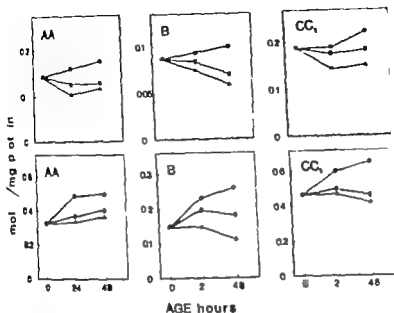
Discussion

Since the purpose of this study was to investigate the role of atmospheric oxygen in regulation of mitochondrial cytochrome content and protein values after birth, optimal and most

TABLE I. Total and mitochondrial protein concentration in heart and liver. Values are means \pm S.D. from 4-8 determinations.

Age	Liver		Heart	
	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.	Total mg/g wet wt.	Mitochondrial mg/g tissue wet wt.
Newborn	131.2 \pm 23.9	39.7 \pm 3.6	109.4 \pm 22.0	26.6 \pm 6.5
1 day	139.3 \pm 19.0	36.6 \pm 6.0	99.3 \pm 10.0	23.5 \pm 6.2
2 days	137.7 \pm 21.3	49.9 \pm 15.9	105.3 \pm 22.1	31.2 \pm 5.6
7 days	160.5 \pm 7.8	55.6 \pm 12.1	121.3 \pm 12.1	38.5 \pm 7.5
14 days	127.7 \pm 26.5	73.3 \pm 13.3	124.1 \pm 12.1	49.7 \pm 8.2
Adult	307.7 \pm 21.5	71.5 \pm 4.8	173.7 \pm 15.3	80.7 \pm 13.1
1 day (starved)	145.5 \pm 15.0	36.8 \pm 7.2	94.3 \pm 7.0	26.9 \pm 4.3
1 day (hypoxic)	136.7 \pm 12.0	26.3 \pm 6.8	104.6 \pm 17.9	23.3 \pm 5.6
2 days (starved)	163.2 \pm 14.9	44.6 \pm 12.4	122.6 \pm 27.8	31.3 \pm 6.7
2 days (hypoxic)	155.8 \pm 29.8	4.9 \pm 8.4 ^a	138.6 \pm 35.7	20.5 \pm 4.7 ^b

^a Significantly lower value ($p < 0.01$) than in starved animals and in control animals of same age.
^b Significantly lower value ($p < 0.05$) than in starved animals and in control animals of same age.



1. Mitochondrial cytochrome concentrations in rat heart (lower pictures) and liver (upper pictures) fed ($\circ-\circ$), starved ($\square-\square$) and hypoxic ($\Delta-\Delta$) rats during the first 48 days after birth. Values are of 6-18 determinations.

the experimental conditions would have been reached if the animals had been born in a gas chamber. In Wistar rats this did not meet with success, and simply because of loss of the newborn by the mother. Perhaps some other more composed strain might be born more suitable. In this case the newborn animals were in normal atmosphere for a time, though for as short a time as possible.

As results show that protein concentration both in the total homogenate and in isolated mitochondria increased in both control and starved animal after birth. Because mitochondrial protein also after the hypoxic periods remained significantly lower than in fed animals, it seems that oxygen lack had primarily an effect on the development of mitochondrial proteins after birth. By comparing the results of the determination of cytochrome and mitochondrial protein concentration in the tissues, an interesting observation can be made, *viz.* the mitochondrial protein concentration was not influenced by fasting as the cytochrome concentration during the postnatal period. However a significant rise in the concentration of mitochondrial protein concentration in the tissue was noted. Dehydration of the fasting animals caused a small degree of increase in the tissue protein concentration, as is evident in Table I. This effect, however, does not interfere with interpretation of the results, *i.e.* this effect does not influence the difference between the fed and hypoxic-fasting animals.

When newborn rats have been maintained in hypoxia for 12 h (10-11% O_2) no increase in cytochromes c or the rate of mitochondrial amino acid incorporation has been found (Haffman 1971), though mitochondrial protein and cytochrome content increases postnatally in normal conditions (Haffman 1971; Haffman *et al.* 1972; Chepelin and Rodriguez

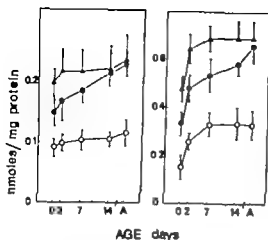


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Mitochondrial protein in the tissues in the hypoxic groups remained significantly lower than in the control group or in the starved group (Table I). Concerning the mitochondrial cytochrome content from one- and two-day-old animals, it could be noticed that in both tissues greatest concentrations were obtained in the control group. After the hypoxic period of 24 h the cytochrome content in both tissues was lower than in the fed or fasting control groups. These differences remained qualitatively the same after the 48-hour observation period, the inhibition of the cytochrome induction was largest in the hypoxic groups, but an inhibition of a smaller extent occurred also in the fasting control group (Fig. 2).

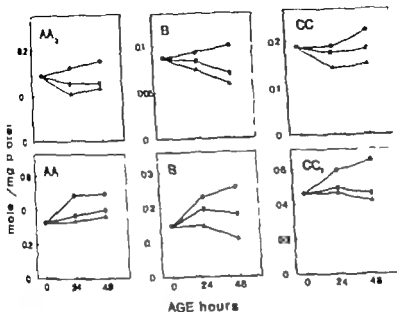
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7 days	160.5 \pm 7.8	55.6 \pm 1.1	121.1 \pm 12.1	32.5 \pm 7.5
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2 days (hypoxic)	155.8 \pm 29.8	4.9 \pm 8.4 ^a	118.6 \pm 35.7	70.5 \pm 4.7 ^b

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Mitochondrial cytochrome concentrations in fat heart (lower pictures) and liver (upper pictures) of (●-●), starved (■-■) and hypoxic (▲-▲) rats during the first 48 days after birth. Values are of 6-10 determinations.

Experimental conditions would have been reached if the animals had been born in a chamber. In Wistar rats this did not meet with success, and simply because of the care of the newborn by the mother. Perhaps some other more composed strain might be more suitable. In this case the newborn animals were in normal atmosphere for time, though for as short a time as possible.

Results show that protein concentration both in the total homogenate and in isolated mitochondria increased in both control and starved animals after birth. Because mitochondrial protein after the hypoxic periods remained significantly lower than in control animals, it seems that oxygen lack had primarily an effect on the development of mitochondrial proteins after birth. By comparing the results of the determination of cytochrome and mitochondrial protein concentration in the tissues, an interesting observation can be made, i.e. the mitochondrial protein concentration was not influenced by fasting as the cytochrome concentration during the postnatal period. However a significant rise in the concentration of mitochondrial protein concentration in the tissue was observed. Dehydration of the fasting animals caused a small degree of increase in the tissue protein concentration, as is evident in Table I. This effect, however, does not interfere with interpretation of the results, i.e. this effect does not influence the difference between the control and hypoxic-fasting animals.

When newborn rats have been maintained in hypoxia for 12 h (10-11% O₂) no increase in cytochromes or the rate of mitochondrial amino acid incorporation has been found (Hannan 1971), though mitochondrial protein and cytochrome content increases postnatally in normal conditions (Hallman 1971, Hallman *et al.* 1972, Chapiro and Rodriguez

de Lores Arnalz 1970 Lisková *et al* 1974) In the studies of Hallman (1971) as well. In the present study oxygen lack inhibited the development of mitochondrial cytochrome after birth. However the present results emphasize that not only the cytochrome but also the total mitochondrial mass as reflected by the mitochondrial protein are regulated by the ambient oxygen tension in mammals.

These results are in full agreement with those obtained from lower animals or mammalian fibroblasts in low oxygen tension (Biggs and Linnane 1963 Wimpenny *et al* 1963 White 1964 Pious 1970). In addition, Park *et al* (1973) with cyanotic dogs and Kinnula (1974) with hypoxic rats have obtained decreased concentration of mitochondrial cytochromes. On the basis of this study it seems probable that during postnatal development environmental oxygen tension is an important factor that regulates the synthesis of mitochondrial proteins after birth.

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The Effect of 6-Aminonicotinamide Blockade of the Pentose Phosphate Pathway on Catecholamines in the Rat Adrenal Medulla, Superior Cervical Ganglion, Hypothalamus and Synaptosome Fractions

By

S.-E. JÄNSSON, J. GRIMENBERG and M. HÄRKÖNEN

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Abstract

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Effect on tissue catecholamines of blockade of the pentose phosphate pathway with 6-aminonicotinamide (6-AN) was studied in the rat. 6-AN at 35-50 mg kg⁻¹ permanently lowered the adrenaline content of adrenal gland to less than 10% of control values and caused 50% loss of noradrenaline, both in control and in stress-treated rats. When the stress tolerance rate was increased by preceding period of stress, 6-AN also markedly depressed noradrenaline in the gland. 6-AN was without significant effect on the noradrenaline content in heart tissue, hypothalamus and superior cervical ganglion and did not affect the uptake or release of catecholamines *in vitro*. The possibility is discussed that 6-AN interferes with the biosynthesis of adrenaline, when it blocks the pentose phosphate pathway by decreasing the supply of reducing equivalents in the form of NADPH which are necessary for the tetrahydropteridine cofactors of tyrosine hydroxylase.

With 6-Aminonicotinamide: pentose phosphate pathway, amine uptake, storage, release, adrenaline, phenylethylamine.

tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis (Lowitt *et al* 1965) requires tetrahydropteridines as cofactors (Nagatsu, Lowitt and Udenfriend 1964) and are kept in a reduced state in a reaction involving NADPH. As the formation of NADPH in neural tissue takes place largely by the pentose phosphate pathway (Härkönen & Kaufman 1974), glucose metabolism via this pathway may be of importance for the maintenance of tyrosine hydroxylase activity. This possibility could be tested with 6-aminonicotinamide (6-AN). In the rat, administration of 6-AN results in the formation of the amino analogue of NADP which, by inhibiting 6-phosphogluconate dehydrogenase, blocks the pentose phosphate pathway (Herken, Lange and Kolbe 1969). If this pathway is

of importance for tyrosine hydroxylase, the 6-AN-induced block would result in loss of tissue catecholamines. It has indeed been shown that 6-AN lowers the adrenal catecholamine content, but this effect was thought to be due to reduced granular uptake of catecholamines (Schacht, Schultz and Senft 1966).

In the present paper the effects of 6-AN on the uptake, storage and release of catecholamines in various neural tissues of the rat have been investigated. The results to be reported indicate that 6-AN has no effect on noradrenaline in the superior cervical ganglion and hypothalamus but that adrenal catecholamines are lowered by 6-AN through a mechanism which partially depends on the turnover rate of the catecholamines.

Methods

Adult Sprague-Dawley rats of both sexes weighing 180–220 g were used. When the effects of 6-AN on tissue catecholamine levels were studied, animals were injected with 6-AN during the morning hours and kept under observation for up to 24 h in a laboratory surroundings with free access to water but without food. 6-AN was administered intraperitoneally in saline, while controls received a saline injection. Tissue specimens were taken in urethane anaesthesia, quenched in liquid nitrogen and stored on CO₂ before determination of catecholamines. The hypothalamus was dissected according to Glowinski & Jervan (1966). The weights of the hypothalamus in the control and 6-AN-treated animals did not differ (60.0 ± 1.5 and 59.5 ± 3.0 mg, respectively). The tarsi of the heart were dissected, soaked in saline and dried by blotting before they were frozen. The adrenal glands were freed from perirenal fat and the superior cervical ganglia were dissected free from the adhering connective tissue. The mean weight of an adrenal gland in a control animal was 20 ± 1.6 mg. Twelve hours after injection of 6-AN (35 – 50 mg kg⁻¹) the weight of the adrenals had decreased to 112% of the control value, but the difference was not statistically significant ($p > 0.1$ and 0.4 , 2 expts., respectively).

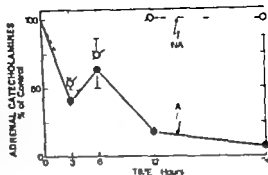
Drum stress. Rats were exposed to drum stress by placing the animals in a 40 cm wide drum with diameter of 56 cm and which rotated at 3 rpm. The drum stress started at 19.00 and lasted for 14 h. At the end a few rats were killed for evaluation of the immediate effect on tissue catecholamines. The remaining animals were divided into 2 groups: one received 6-AN at 35 mg kg⁻¹ i.p. the other saline. After the injection, the animals were allowed to recover with free access to water but without food. The replenishment of tissue catecholamines was followed for 24 h.

Catecholamine uptake and efflux experiments. Adrenal slices, 360 μ m thick were prepared with a Thomas tissue chopper. The slices were incubated in a physiological electrolyte medium (see below) buffered at pH 7.4 with carbonyl buffer. Pinched-off nerve ends (synaptosomes) were isolated from whole brain homogenates by density-gradient centrifugation (Ficoll-sucrose gradient) and incubated in a Tris-HCl buffered medium (to be described elsewhere) in preparation.

In the amine uptake experiments, synaptosomes were preincubated for 5 min at 37°C. Thereafter 11- β -noradrenaline at a specific activity of 0.64 Ci mmol⁻¹ was added to the incubation system consisting of 1 ml incubation medium supplemented with 0.2 mg ml⁻¹ ascorbic acid, 10^{-6} mol l⁻¹ naloxone and 1 mg ml⁻¹ synaptosomal protein (proteins determined according to Lowry *et al.* 1951). The final amine concentration was 1.5×10^{-6} mol l⁻¹. Uptake was terminated by vacuum filtration through a Millipore filter (pore size 0.45 μ m, diameter 25 mm) and the filter and synaptosomes were washed with 2 changes of fresh incubation medium ($3 + 7$ ml). The filters were scintillation counted in 10 ml of Bray's liquid scintillation medium (1960), and counted in a LKB-Wallac 81000 liquid scintillation counter. During the first 5 min at 37°C the uptake was approximately linear and between 0.3 and 1.6 mg ml⁻¹ was proportional to the protein concentration in the medium. Amine uptake was expressed as pmol g⁻¹ protein and was corrected for zero-time uptake obtained by interrupting the uptake process by filtration and washing immediately after addition of the radioactive substrate to the system.

In some experiments, the efflux of amines was followed. Adrenal slices were preloaded with 11- β -noradrenaline (specific activity 14 Ci mmol⁻¹, final concentration, 7×10^{-6} mol l⁻¹) for 30 min at 37°C. After 3 washes, the slices were reincubated with or without added 6-AN. At various times, samples of the incubation medium were transferred to bottles containing 10 ml of Bray's solution and counted. The counting efficiency determined with 11- β -tetradecane as internal standard was 4%.

Fig. 1 Effect of 6-AN on adrenal catecholamines. 6-AN was injected i.p. at 35 mg kg⁻¹. Values are expressed as per cent of the controls killed at appropriate time intervals after the injection. Each point is a mean \pm S.E. for 4-8 rats. A—adrenaline, NA—noradrenaline.



Determination of 6-phosphogluconate (6-PG) 6-Phosphogluconate was measured fluorometrically by the method of Koeffler *et al.* (1949).

Determination of glucose The adrenal slices, contained in a small net-basket in stainless steel tin, were incubated in 50-ml centrifuge tubes in the incubation medium containing 5.5 mmol l⁻¹ glucose for 1 min. Thereafter the basket was transferred to another centrifuge tube containing glucose-free incubation medium. After rapid runs in this medium the basket was pulled up to hang in the tube out of contact with the incubation solution. The outlet of the gas tubing was closed and O₂-CO shut off. At zero-time, 1 min and 20 min some of the slices were rapidly sampled, frozen in liquid N₂ and assayed for glucose (Baker, Pennocks and Lowry 1949).

Determination of catecholamines Tissue samples were homogenized and extracted according to Auer and Sayre (1962) in 0.2 mol l⁻¹ phosphate buffer pH 7.8, which contained 24 mg p-chloromercuriphenyl-sulphonic acid per g tissue and 10 mg ml⁻¹ EDTA. The fluorometric determination of adrenaline and noradrenaline was by the trihydroxyindole method (Auer and Sayre 1962, Rich-Malherbe 1963). The values of noradrenaline and adrenaline are calculated from fluorescence readings at 285 nm (lengths 28.45 and 43.510 nm, respectively uncorrected instrumental readings) using an Aminco-Bowman spectrofluorometer. The recovery from external standards is 73.1 \pm 3.3 and 81.0 \pm 2.2 for noradrenaline and adrenaline, respectively. Values were expressed in nmol or μ mol per g tissue or per mg wet weight and refer to free bases corrected for blank readings and recoveries.

The significance of differences between means was determined using the *t*-test.

Solutions and drugs The incubation medium had the following molar composition: NaCl, 136 g; 5.6, MgCl₂, 1.1, CaCl₂, 2.2, PO₄, 1.3, glucose, 5.5 and bovine serum albumin at 1 mg ml⁻¹. The pH was adjusted to 7.4 with 20 mmol l⁻¹ Tris-HCl buffer. The medium was buffered to pH 7.4 with 20 mmol l⁻¹ Tris-HCl buffer. The medium was bubbled with gas mixture of 85% O₂ and 15% CO₂. H-noradrenaline and H-adrenaline specific activities of 0.64 Ci mmol⁻¹ and 1.4 Ci mmol⁻¹ respectively were obtained from the New England Nuclear Corporation. 6-AN was obtained from the Sigma Chemical Company.

Results

Effect of 6-AN on adrenal catecholamines The concentration of noradrenaline and adrenaline in the adrenal glands of control animals was 0.588 \pm 0.053 and 3.36 \pm 0.33 μ mol g⁻¹ respectively (means \pm S.E. of 31 animals). 6-AN administered i.p. at 35-50 mg kg⁻¹ practically depleted the adrenals of adrenaline in 12 h, whereas noradrenaline was only transiently lowered. 3 h after the injection the noradrenaline content was reduced to 55% of the control level. Adrenaline stores were even lower (Fig. 1). After this initial decline, the noradrenaline content was gradually restored, being equal to the control within 1 h. Adrenaline was depleted only to a limited extent; 6 h after the injection, a second phase of depletion started.

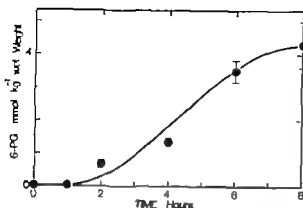


Fig. 2. Accumulation of 6-phosphogluconate (6-PG) in the adrenal medulla after intraperitoneal treatment with 6-AN. 6-AN was injected i.p. at 50 mg kg^{-1} . Each point represents the mean \pm S.E. for 3 animals. The zero-time 6-PG concentration was $16.5 \mu\text{mol kg}^{-1}$.

which brought adrenaline levels down to 18 and 7% of the control values at 12 and 74 h, respectively. In the controls the adrenaline/noradrenaline ratio was 6:1. This ratio was reversed after treatment with 6-AN.

The first phase of 6-AN-induced amine depletion in the adrenals took place when the pentose phosphate pathway was still operative, whereas the second phase of adrenaline depletion did not occur until metabolism via the pathway was blocked. This was shown by following the accumulation of 6-phosphogluconate in the adrenal gland after injection of 6-AN at 50 mg kg^{-1} . After 3 h, 6-phosphogluconate levels started to increase and reached a near maximum of $3.52 \text{ mmol kg}^{-1}$ after 6 h (Fig. 2). This increased value should be compared with the endogenous level of $16.5 \mu\text{mol kg}^{-1}$ in control glands (210-fold increase).

Effect of 6-AN on noradrenaline in hypothalamus and atrial tissue. The drastic effects of 6-AN on adrenal catecholamines seem to be specific to this tissue. No significant effects on noradrenaline stores were observed in hypothalamus or heart tissue (Table 1).

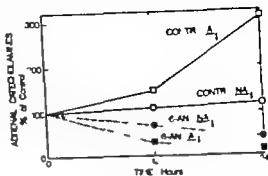
Effect of 6-AN on adrenal catecholamines after drum stress. Immediately after a 14 h period of drum stress, the noradrenaline and adrenaline contents of the adrenal glands fell to 66 and 31% of the respective control values. In the animals which received only a saline injection after cessation of the drum stress, resynthesis of adrenal catecholamines proceeded during the first 24 h at a rate of $49.2 \text{ nmol g}^{-1} \text{ h}^{-1}$. The half life of catecholamine restoration in the adrenal gland was 20.4 h (calculated from the rate constant of total amine resynthesis):

TABLE 1. Effect of 6-AN on noradrenaline stores in the hypothalamus and atria.

Time after injection of 6-AN (hours)	Noradrenaline,	of control
	Hypothalamus	Atria
	74.0 ± 12.9	102.6 ± 11.0
4	130.5 ± 26.7	84.8 ± 9.1
6	119.0 ± 7.6	—
10	79.5 ± 3.4	79.9 ± 4.6

6-AN was injected i.p. at 50 mg kg^{-1} . Values are expressed as percentages of those for controls injected with saline and killed after the same time intervals. Means \pm S.E. for 3 rats. Pooled control values were $10.4 \pm 1.2 \text{ nmol g}^{-1}$ and $10.8 \pm 1.1 \text{ nmol g}^{-1}$ for hypothalamus and atria, respectively.

Fig. 3 Effect of 6-AN on adrenal catecholamines after drum stress. After cessation of the drum stress, the animals were killed into 2 groups. One group received 6-AN p at 35 mg kg^{-1} while the other acted as saline controls. Values are expressed as percentages of the catecholamine secretion after the cessation of the drum stress (adrenaline, 0.614 and noradrenaline, $0.347 \mu\text{mol g}^{-1}$). Each point represents the mean for 3-9 animals. A: adrenaline, NA: noradrenaline.



this corresponds to a turnover time of 5.1 days, which is of the same order of magnitude as was obtained previously (Eränkö and Härkönen 1961). The rate of adrenaline recovery was 6.7 times as rapid as that of noradrenaline. These events are illustrated on a normalized scale in Fig. 1.

When administered immediately after the drum stress ceased, 6-AN effectively prevented restoration of noradrenaline and adrenaline stores in the adrenal glands. At 12 h after the injection of 6-AN at 35 mg kg^{-1} the adrenaline content was only 32 % of the value found immediately after the drum stress, and after 24 h the adrenals were almost depleted of adrenaline (Fig. 3). Likewise, noradrenaline stores fell on treatment with 6-AN the fall being continuous instead of transient as it was in unstressed animals. Twenty-four hours after the injection, the noradrenaline concentration in the adrenal gland was reduced to $0.138 \mu\text{mol g}^{-1}$ as compared with $0.347 \mu\text{mol g}^{-1}$ immediately after drum stress. During this period, the noradrenaline stores in the saline-injected animals had increased to $0.399 \mu\text{mol g}^{-1}$.

Effect of 6-AN on noradrenaline in hypothalamus and cervical ganglion after drum stress
The noradrenaline content in the superior cervical ganglion was not affected by the drum stress while in the hypothalamus, noradrenaline stores showed an insignificant increase to 108 ($p > 0.2$) of the control value (Table II).

After drum stress, 6-AN was without significant effect on noradrenaline stores in the superior cervical ganglion (Table III). In the hypothalamus, a 30 % decrease in the noradrenaline content was observed 24 h after the 6-AN injection (35 mg kg^{-1}), but the difference was not statistically significant ($p > 0.3$).

Table II Effect of drum stress on the noradrenaline concentration in the superior cervical ganglion and hypothalamus

	Noradrenaline concentration	
	Cervical ganglion (nmol per ganglion)	Hypothalamus (nmol g ⁻¹)
Control	0.30 ± 0.04	8.70 ± 0.80
Drum stress	0.30 ± 0.07	11.00 ± 2.05

Drum stress was carried out as explained in Methods. Means \pm S.E. for 9 (controls) or 6 (drum stress) animals.

TABLE III Effect of 6-AN on the noradrenaline concentration in the hypothalamus and cervical ganglion after drum stress.

Time after drum stress and 6-AN injection (hours)	Noradrenaline content			
	Cervical ganglion (nmol per ganglion)		Hypothalamus (nmol g ⁻¹)	
	Control	6-AN	Control	6-AN
12	0.53 ± 0.10	0.62 ± 0.10	10.7 ± 2.0	10.0 ± 1.7
24	0.63 ± 0.03	0.63 ± 0.06	11.4 ± 1.7	8.0 ± 1.4

Immediately after the cessation of the drum stress, 6-AN was injected i.p. at 35 mg kg⁻¹. Means ± S.E. for 4-6 animals.

Effect of 6-AN on adrenal adrenaline content in vitro 6-AN did not affect adrenaline stores in the gland *in vitro*. This is shown in Fig. 4 where the efflux of ³H from slices pre-loaded with ³H-adrenaline took place at the same rate whether the slices were treated with 6-AN or not.

Effect of 6-AN on the uptake of noradrenaline by synaptosomes When synaptosomes were treated with 10⁻⁴ mol l⁻¹ 6-AN at room temperature 6-phosphogluconate began to accumulate immediately. At 1 and 2 h after the addition of 6-AN the level of 6-phosphogluconate in synaptosomes was increased by 4 and 6 times respectively (Fig. 5). Despite this pronounced rise, 6-AN did not affect the uptake of ³H-noradrenaline by the synaptosomes (Fig. 6).

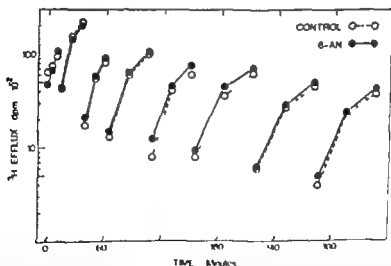
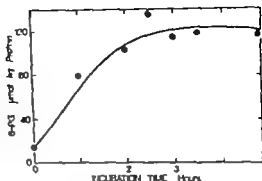


Fig. 4 Effect of 6-AN on the efflux of ³H from adrenal slices after preloading with ³H-adrenaline. Adrenal slices were incubated with ³H-adrenaline as explained in Methods. After incubation the slices were divided into 2 groups: to one 6-AN was added at 10⁻⁴ mol l⁻¹ while the other served as control. Incubation was continued at room temperature for up to 5.75 h. The incubation volume was 1.0 ml and 10 min after the addition of 6-AN, 200 µl samples of the incubation medium were removed for the determination of ³H-efflux. Extracellular accumulation and subsequently accelerated reuptake of ³H-adrenaline were avoided by aspirating the incubation medium at predetermined intervals and replacing it with fresh medium. In the figure, this is indicated by the regularly spaced intervals between each 3-sample portion of the efflux curve. Results of 1 experiment.

5. Accumulation of 6-phosphogluconate
 G. synaptosomes after treatment with
 6-AN. Synaptosomes are incubated
 with 6-AN at 10^{-4} M.
 Each point represents the result of
 4 experiments.



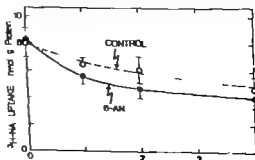
did 6-AN affect synaptosomal ^3H -noradrenaline uptake when administered *in vitro*. Mice were injected with 6-AN at 50 mg kg^{-1} and killed 3 h later; the synaptosomes isolated took up $10.95 \pm 0.44 \text{ nmol noradrenaline g}^{-1} \text{ protein min}^{-1}$ as compared with the control value of $11.39 \pm 0.53 \text{ nmol g}^{-1} \text{ protein min}^{-1}$ (means \pm S.E. of 4 expts.).

Discussion

Results confirm the observation by Schacht *et al.* (1966) that 6-AN administered during stress conditions depletes adrenaline stores in the adrenal gland *in vivo* whereas noradrenaline is only transiently lowered. These effects are seen only in the adrenals and may be due to a specific drug effect or could be secondary to the toxic effects of the drug. 6-AN causes diabetes (see Leao 1970), a condition which increases the adrenal catecholamine turnover but does not cause any actual depletion of the amines (Leduc 1961; Gordon *et al.* 1969). The toxic effects of 6-AN include extrapyramidal-motor disorders which, by constituting a stress situation, might hypothetically lower adrenal amine stores. However motor signs were not manifested until about 4 h after the 6-AN injection, while the amine is still high. Although an effect on adrenal catecholamine stores due to a 6-AN-induced stress situation cannot be excluded, specific drug effects appear more probable.

The decrease in adrenal catecholamines induced by 6-AN *in vivo* appears to result from peripheral effects. The initial 50% reduction is most likely explained by an effect of 6-AN on the adrenal nerves or directly on the medullary cells, since at this time the pentose

6. Effect of 6-AN on the uptake of ^3H -noradrenaline by synaptosomes *in vitro*. Synaptosomes were incubated with 6-AN at 10^{-4} M at room temperature. At various times after the addition of 6-AN, the ability of synaptosomes to take up ^3H -noradrenaline from the medium was tested as described in Methods. Each point is the mean \pm S.E. of 4-10 expts.



phosphate pathway was still operative. The mechanism underlying this initial 6-AN effect remains unclear. The drug is hardly likely to affect amine uptake or storage or release mechanisms, as these parameters remained unaffected in adrenal slices and synaptosomes treated with 6-AN. However, the initial amine depletion observed after 6-AN appears to be specific for the adrenal glands, may be reversible and is known to occur with a simultaneous loss of ATP (Schacht *et al.* 1966). In this respect it differs from the subsequent phase of depletion which is characterized by a low amine:ATP ratio (Schacht *et al.* 1966).

The later sustained effect of 6-AN on adrenal amine stores occurred at the time when blockade of the pentose phosphate pathway was established, and exhibited an apparent selectivity for adrenaline. The reason for this is unknown but during resting conditions the increased amine synthesis due to reduced end-product inhibition (Nagatsu *et al.* 1964, Udenfriend *et al.* 1965, Stjärne 1966) allowed complete restoration of noradrenaline stores.

Even when the pentose phosphate pathway was blocked by 6-AN, amine uptake and release processes remained unaffected in synaptosomes and adrenal slices. These negative *in vitro* results would be understandable if the effects induced by 6-AN depend on intact innervation. Present results indicate that this may be the case: during resting conditions, 6-AN only transiently lowered noradrenaline stores in the adrenal gland, while sustained depletion took place when the amine turnover rate was increased by a preceding period of drug stress. Tetrahydropteridines act as cofactors of tyrosine hydroxylase (Nagatsu *et al.* 1964) and are maintained in a reduced state in a reaction involving NADPH. As the NADPH in neural tissue is largely formed via the pentose phosphate pathway (Härkönen and Kauffman 1974), and as tyrosine hydroxylase is the rate limiting step in catecholamine biosynthesis (Lewitt *et al.* 1965), blockade of the pathway could result in retarded amine synthesis and loss of tissue catecholamines when the turnover rate is increased during a stress situation.

The supposed inhibition of tyrosine hydroxylase after blockade of the pentose phosphate pathway resulted in amine loss only in the adrenals and not in the superior cervical ganglion or in the hypothalamus. This might reflect differences in ability to reaccumulate secreted catecholamines, with the exception of the adrenal medulla, in which reuptake is low (see Stjärne 1972). Neural tissue has a good ability for reuptake of amines (Haefely, Hürlimann and Thoenen 1965). However, blockade of the pentose phosphate pathway would be expected to produce pronounced effects especially in the adrenals since in this tissue, metabolism via the pathway is exceptionally active. If it is assumed that the linear increase in 6-phosphogluconate concentration 3 h after injection of 6-AN was due to complete inhibition of 6-phosphogluconate dehydrogenase, and that the oxidation of glucose-6-phosphate was the only source of 6-phosphogluconate, the rate at which glucose would enter the pentose phosphate pathway would be $0.91 \text{ mmol kg}^{-1} \text{ wet weight h}^{-1}$. This value corresponds to approx. 37% of the normal hexose use in adrenal medullary slices ($2.44 \text{ mmol kg}^{-1} \text{ wet weight h}^{-1}$), which is considerably higher than estimates of the pentose phosphate pathway activity in brain (3–5%, Hostetler *et al.* 1970) and in superior cervical ganglion (16%, Härkönen and Kauffman 1974).

We conclude that NADPH generated in the pentose phosphate pathway may be important for the maintenance of the tyrosine hydroxylase activity needed for adequate biosynthesis

catecholamines during conditions of increased amino utilization. A closer study of the effect of 6-AN on amine turnover rates is indicated.

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phosphate pathway was still operative. The mechanism underlying this initial 6-AN effect remains unclear. The drug is hardly likely to affect amine uptake or storage or release mechanisms, as these parameters remained unaffected in adrenal slices and synaptosomes treated with 6-AN. However, the initial amine depletion observed after 6-AN appears to be specific for the adrenal glands, may be reversible and is known to occur with a simultaneous loss of ATP (Schacht *et al* 1966). In this respect it differs from the subsequent phase of depletion which is characterized by a low amine:ATP ratio (Schacht *et al* 1966).

The later sustained effect of 6-AN on adrenal amine stores occurred at the time when blockade of the pentose phosphate pathway was established, and exhibited an apparent selectivity for adrenaline. The reason for this is unknown but during resting conditions the increased amine synthesis due to reduced end product inhibition (Nagatsu *et al* 1964, Udenfriend *et al* 1965, Stjärne 1966) allowed complete restoration of noradrenaline stores.

Even when the pentose phosphate pathway was blocked by 6-AN, amine uptake and release processes remained unaffected in synaptosomes and adrenal slices. These negative *in vitro* results would be understandable if the effects induced by 6-AN depend on intact innervation. Present results indicate that this may be the case: during resting conditions, 6-AN only transiently lowered noradrenaline stores in the adrenal gland, while sustained depletion took place when the amine turnover rate was increased by a preceding period of drug stress. Tetrahydropteridines act as cofactors of tyrosine hydroxylase (Nagatsu *et al* 1964) and are maintained in a reduced state in a reaction involving NADPH. As the NADPH in neural tissue is largely formed via the pentose phosphate pathway (Härkönen and Kauffman 1974), and as tyrosine hydroxylase is the rate limiting step in catecholamine biosynthesis (Lewitt *et al* 1965), blockade of the pathway could result in retarded amine synthesis and loss of tissue catecholamines when the turnover rate is increased during a stress situation.

The supposed inhibition of tyrosine hydroxylase after blockade of the pentose phosphate pathway resulted in amine loss only in the adrenals and not in the superior cervical ganglion or in the hypothalamus. This might reflect differences in ability to reaccumulate secreted catecholamines, with the exception of the adrenal medulla, in which reuptake is low (see Stjärne 1972); neural tissue has a good ability for reuptake of amines (Härfely, Hürmann and Thoenen 1965). However, blockade of the pentose phosphate pathway would be expected to produce pronounced effects especially in the adrenals since in this tissue, metabolism via the pathway is exceptionally active. If it is assumed that the linear increase in 6-phosphogluconate concentration 3 h after injection of 6-AN was due to complete inhibition of 6-phosphogluconate dehydrogenase, and that the oxidation of glucose-6-phosphate was the only source of 6-phosphogluconate, the rate at which glucose would enter the pentose phosphate pathway would be $0.91 \text{ mmol kg}^{-1} \text{ wet weight h}^{-1}$. This value corresponds to approx. 37% of the normal hexose use in adrenal medullary slices ($2.44 \text{ mmol kg}^{-1} \text{ wet weight h}^{-1}$), which is considerably higher than estimates of the pentose phosphate pathway activity in brain (3–5%, Hostetler *et al* 1970) and in superior cervical ganglion (16%, Härkönen and Kauffman 1974).

We conclude that NADPH generated in the pentose phosphate pathway may be important for the maintenance of the tyrosine hydroxylase activity needed for adequate biosynthesis

synth 1971; Daniels *et al.* 1974). Furthermore, the very low concentrations of Ca²⁺ and iron in the adrenal vesicles (Borowicz *et al.* 1965), as well as infrared (Pal and Maynert 1967) and electromagnetic (Daniels *et al.* 1974) spectra of medullary granule material argue for a role for metal ions in an ATP-CA storage complex.

Assuming an ATP:CA ratio of 1:4 in the ATP-CA storage complex we have still left in the storage of the remaining 20–30% of the CA in the adrenal medulla which

is loosely linked to the phosphate groups of ATP.

Isophilic granules of mast cells have a high amine binding capacity both *in vitro* and *in vivo* (Uvnäs *et al.* 1970; Uvnäs and Bergendorff 1973). From a storage point of view these granules contain negligible amounts of ATP. Their matrix consists of a heparin-protein complex with the properties of a weak cation exchanger material, capable of binding both acidic cations and biogenic amines. Experimental evidence indicates that endogenous histamine is stored in ionic linkage to free carboxyls in the protein part of this granule complex and is released by cation exchange when these ionic sites become exposed to cellular cations (mainly sodium) during the exocytosis of the mast cell granules (for review see Uvnäs 1975).

In the present study the ability of bovine adrenal medulla granule material which had been depleted of CA and ATP by dialysis to bind inorganic and organic cations has been studied. The material has the properties of a weak cation exchanger and the binding capacity suffices to explain the storage of 20–30% of the normal CA content of the bovine adrenal granules.

Methods

Isolation of granule material from adrenal medulla

Isolated medullary granules were isolated according to Foweraker and Trifaro (1967). The adrenals were taken from the slaughter house on ice and immediately on arrival (about 3 h after death) the medulla was removed and homogenized in ice-cold 0.32 M sucrose using glass homogenizer. After centrifugation at 800 g the supernatant was removed and filtered under ~100 p. nitrogen pressure through a series of 25 µm nuclepore filters placed in a Sartorius filter apparatus, type SM 16223. The pore sizes were 1.2, 0.6, 0.45 and 0.2 µm. After passing the 0.2 µm filter the filtrate was centrifuged for 10 min at 20 000 g and the resulting sediment was suspended in 4 ml of ice-cold deionized water. This material was then dialyzed against 4 l of deionized water under continuous rocking for 24 h. All the dialyses described above were performed in the cold room (4 ± 0.5°C). The dialyzed material, found to be almost completely depleted of CA and ATP, was diluted with deionized water to 7.5–9.5 ml before use in the plate studies. This suspension will be referred to below as the granule suspension.

Media

Media at pH 7.4 Fresh 0.2 M sodium phosphate buffer was prepared by titrating 20 ml of 1 M sodium concentrated H₂PO₄ to ~pH 7.3, and diluted to 100 ml with deionized water. The pH was adjusted to 7.30 with conc. H₂PO₄. From this 0.2 M buffer stock solution 9 ml samples were prepared containing 200, 60, 20, 6, 2, 0.6 and 0.2 µmol of Na⁺/ml. To each sample 25 µl of ³H-NaCl (100 µCi/ml) was added. 1 ml of each of the samples was added to 1 ml of granule suspension and the pH was adjusted to 7.4 with 1 M NaOH. The NaOH added was taken into account when calculating the uptake of catecholamines by the granule material.

1 ml samples of granule material suspended in sodium phosphate buffer were filtered through a 0.2 µm filter (UM 2, Daclo, Amicon, 25 mm) in the Sartorius filter apparatus under nitrogen pressure. The UM 2 filter was stated to retain materials with molecular weights above 1 000. The filtrate at pH ~7.4 (at room temperature). The UM 2 filter with its moist layer of retained granule material was removed from the filter apparatus and folded manually to fit the opening of a tapered glass tube (11 × 55 mm).

The Ability of ATP Free Granule Material from Bovine Adrenal Medulla to Bind Inorganic Cations and Biogenic Amines

By

BÖRJE UVNÄS and CARL HUGO ÅBORG

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Abstract

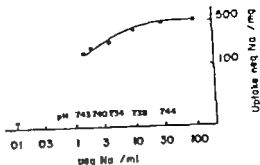
UVNÄS, B. and C. H. ÅBORG. *The ability of ATP free granule material from bovine adrenal medulla to bind inorganic cations and biogenic amines*. Acta physiol. scand. 1977 99: 476-483.

Bovine adrenal medullary granules isolated by millipore filtration were depleted of CA and ATP by dialysis. The resulting material showed an ability to bind inorganic cations and biogenic amines in a concentration-dependent manner. The similarity of the uptake curves, the identical uptake maxima and the narrow pH range (between 4-7) over which the uptake of the inorganic and organic cations took place indicated binding of these ions to common sites. In addition, the fact that all the uptake curves fitted the Rothman-Kornfeld equation for cation exchangers corroborated the cation exchanger properties of the dialyzed granule material. The CA binding capacity corresponded to 70-80% of the normal CA content of bovine medullary granules.

The concomitant occurrence in adrenal medullary granules of catecholamines (CA) and adenosine nucleotides, mainly adenosine-5-triphosphate (ATP) has led to the widely accepted hypothesis that the CA are stored in the granules in some kind of a complex with this nucleotide. Since at pH 7.4 CA are positively charged and ATP carries 4 negative charges, it has been suggested that CA are ionically bound to the polyvalent nucleotide (Blaschko *et al* 1956, Falck *et al* 1956). However, since the ratio—total positive CA charge/total negative nucleotide charge—was found to be ≈ 1 , some other kind of storage mechanism had to be assumed for part of the CA. According to Hillarp (1960), in the bovine adrenal medulla at least 20% of the CA had thus to be stored in a manner not involving ATP.

From comparative physico-chemical studies on aggregates formed *in vitro* between CA and ATP—with or without the admixture of alkaline earth metal ions (Ca^{2+} and Mg^{2+})—and of adrenal medullary granules, Bernets *et al* (1971) concluded that aggregate formation with ATP may play an essential role in the uptake and storage of CA in adrenal medullary granules. Recent studies using high resolution proton and phosphorus electromagnetic spectroscopy were considered to confirm the occurrence in bovine medullary granules of an ATP-CA complex and to support the idea of an ATP-CA ratio of 1:4 in this complex (Pal

Concentration dependent uptake of γ dried granule material from adrenal medulla suspended in sodium phosphate pH 7.4. Observed uptakes \bullet — \bullet calculated for pH 7.4 \circ — \circ from the Rothmund-Kornfeld equation; β 1 K= U_{max} 538 neq/mg.



Results

Uptake of inorganic cations and biogenic amines

Concentration dependence. As illustrated for sodium (Na^+) in Fig. 1 and for noradrenaline (NA) in Fig. 2, all the inorganic cations (Na^+ , Ca^{++}) and biogenic amines (PhEA, TA, NA, HI) studied were taken up from the suspension medium by the granule material. The uptake was observed to be concentration-dependent within a cation concentration range from 0.01 and 30 meq/ml. The maximal uptake capacity varied somewhat between the kinds of the granule material used, in most cases it was between 400 and 500 neq/mg dry light.

pH-Dependence. The cation uptake was strongly influenced by the pH of the suspension medium. Lowering the pH from 7–8 to 4–5 abolished the storage capacity of the granule material, as illustrated for Na^+ and NA in Fig. 3.

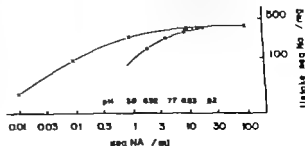
Titration for free acid groups

Titration of granule suspensions with 0.1 M HCl showed a consumption of between 400–500 meq of acid/mg dried material between pHs 7.4 and 5.0. The titration values for each kind of granule material agreed well with its cation uptake capacity.

Application of the Rothmund-Kornfeld equation

The Rothmund-Kornfeld equation (Eq. 1) is an empirical modification of the Law of the Mass Action valid for cation exchangers (Samuelsson 1952). The equation implies that, when two cations compete for the ionic sites of the exchanger, plotting the log of the ratios

Fig. 2. Concentration dependent uptake of NA by dried granule material from adrenal medulla suspended in NA solution at pH ~7. Observed uptakes (—) Uptake calculated (—) for pH 7.4 from the Rothmund-Kornfeld equation β 0.7 K= $1.26 \cdot 10^{-4}$ U_{max} 480 neq/mg.



In order to minimize loss of the material when it was scraped down into the tube (with a stainless steel spatula). The previously weighed tube was reweighed with its moist content, then dried over P_2O_5 *in vacuo* to constant weight (70 h), and again weighed. One ml of 0.4 M HCl was added to the tube which was then closed with a plastic stopper. The stopper was secured with tape and the tube was heated to about 60°C whilst shaking vigorously. After centrifugation for 30 min at 3000 *g* 0.5 ml of the supernatant was neutralized with 0.4 ml of 0.5 M NaOH and 15 ml of scintillation fluid was added (0.4% 2,5-diphenyl-oxazole, 0.01% 1,4-bis-2(4-methyl-5-phenyl oxazolyl)-benzene in a mixture of 50% toluene and 50% ethylene glycol monoethyl ether).

To determine the $^{22}NaCl$ radioactivity in the suspension fluid after its passage through the UM 2 membrane, 0.5 μ l was taken from each sample of the filtrate. These reference samples were dried and treated exactly as described above for the granule material. The determination of the $^{22}NaCl$ activity in the filtrates, together with the (careful) weighing procedures enabled a reliable estimate of the uptake of sodium (and amines) by the granule material to be made. When calculating the uptake, corrections were made for changes in the concentration of sodium (or the amine) in the suspension fluid due to the uptake by the granule material.

Uptake of amines at pH 6-7

Solutions of the amines were titrated to pH 6-7 with 5-6% H_2SO_4 and diluted to the desired concentrations with deionized water in a manner analogous to that described above under sodium uptake. H_2SO_4 was used since it yields water soluble salts, prevents oxidation and has a maximum buffering capacity at pH 7 ($pK_a = 6.9$). Considering these advantages, the drawback of a rather poor buffering capacity was knowingly accepted.

Influence of pH on the uptake of sodium

0.1 M sodium phosphate buffer was prepared by titrating 1 M NaOH with conc. H_3PO_4 to pH 7.2. Equal volumes of granule suspension and buffer solution were mixed and appropriate amounts of $^{22}NaCl$ were added. During stepwise acidification with conc. H_3PO_4 0.5 ml samples were taken at desired pHs for the determination of sodium uptake as described above. To retain constant Na^+ ion concentrations during the titration the H_3PO_4 solution contained 0.05 M sodium chloride and the same concentration of $^{22}NaCl$ as the sample to be titrated.

Influence of pH on the uptake of amines

Solutions of the amines were titrated to pH 7 with 5-6% H_2SO_4 . Appropriate amounts of radioactive amines were added. During stepwise acidification with conc. H_2SO_4 , samples were taken at desired pHs for the determination of amine uptake as described above.

Titration of granule material for free acid groups

2 ml of granule suspension \rightarrow deionized water or 0.1 M $NaCl$ \rightarrow was titrated at 0°C, and at room temperature to pH 9.1 with 0.1 M $NaOH$ and then with 0.1 M HCl to pH 3.0.

To minimize possible disturbing effects of various enzymes and of bacterial growth, all procedures described above, except titration were performed in the cold room (+4°C). A Agla micrometer syringe was used for all titrations.

Materials

^{22}Na (sodium chloride in aqueous solution carrier free, 100 mCi/mg Na), DL (7 H) *adrenaline* (91 mCi/mg), L-(7,8 H) *noradrenaline* (43.5 mCi/mg), *tyramine*-(1 C) *hydrochloride* (314 μ C/mg), β -phenylethylamine-(1 C) HCl (45 μ C/mg), (ring-2 C) *histamine dihydrochloride* (50 mCi/mmol) and ^{45}Ca (calcium chloride in aqueous solution, 10-40 mCi/mg Ca), were obtained from the Radiochemical Centre, Amersham, England.

Abbreviations

A = Adrenaline
NA = Noradrenaline
TA = Tyramine
PhEA = Phenylethylamine

DA = Dopamine
CA = Catecholamine
ATP = Adenosine-5'-triphosphate
Hl = Histamine

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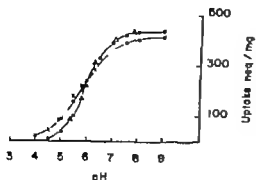


Fig. 3 Influence of pH on the uptake of Na and NA by dialyzed granule material from adrenal medulla. Conc. Na 100 μ eq/ml, NA 70 μ eq/ml. Observed uptakes for Na Δ — Δ and NA \bullet — \bullet . — Uptakes calculated according to the Rothmund-Kornfeld equation from the conc. curves in Fig. 1 and for Na \circ — \circ and for NA^+ \bullet — \bullet respectively.

between the concentrations of the two cations in the exchanger and the log of the corresponding concentrations of the cations in the suspension medium should give a straight line.

Assuming that in the present expts. the granule material behaves like a cation exchanger the two cations competing for the binding sites will be hydrogen ions and the cation under study. Since the maximal uptakes for these two ions in the same batch of granule material are the same, and the pH and the cation uptake are known the validity of the exchanger equation for the cation binding of the granule material can be tested.

As can be seen from Fig. 4 the uptake values obtained for all the inorganic and organic cations studied fit the cation exchange equation. It is noteworthy that the monoamines A, NA, TA and PHEA had practically identical affinities for the binding sites, in contrast to the considerable differences between the monovalent Na and the divalent Ca^{2+} ions.

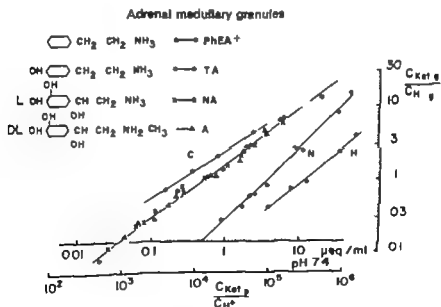


Fig. 4 Plotting of the ratios between inorganic and organic cation concentrations, and hydrogen ion concentrations in dialyzed granule material against the corresponding cation concentration in the suspension medium. For symbol see the figure.

Note: For graphic constructions of concentration and pH curves at pH 7.4 the cation conc. in suspension medium at pH 7.4 (line above the abscissa) is related to the ratio cation conc./hydrogen ion conc. in the suspension medium.

$$\frac{[B_+]}{[A_+]} \left(\frac{[A_+]}{[B_+]} \right)^{\beta} = K \quad (1)$$

1. The Rothmund-Kornfeld equation, A_+ and B_+ = ions in ion exchange material (resin), A_+ and B_+ = solutions, β and K = empirical factors

some seem to have a lower affinity for the granule binding sites than do the other ions studied.

From the concentration or pH curves the empirical factors K and β in the Rothmund-Kornfeld equation can be calculated. The pH curves calculated from the experimentally found concentration curves in Fig. 1 and 2 fit the experimentally obtained curves well (Fig. 3).

In our studies of the concentration-dependence of the uptake of the amines we were able to keep the pH constant at neutrality. With diminishing amine concentrations there is a progressive slight acidification towards pH 6. Due to the strong pH dependence of uptake, even a slight fall in pH resulted in a considerably reduced uptake. This fact is stated in Fig. 2. A recalculation for pH 7.4 according to the Rothmund-Kornfeld equation yields a concentration range for the uptake of NA considerably wider than experimentally obtained.

Discussion

Our present results demonstrate that granule material from bovine adrenal medulla prepared by isopycnic filtration according to Posner and Trifaró (1967) retains an ability to bind organic cations and biogenic amines even after dialysis against water. Dialysis depleted the material of CA and ATP—as determined chemically—and presumably of other diffusible organic and organic materials. In most of our experiments the dialyzed material was used for uptake studies without further purification. Removal of undissolved material, and thereby loss of the insoluble membrane fraction, by high speed centrifugation (100 000 g for 1 min) reduced the uptake capacity of the remaining soluble material to only a minor degree. The uptakes of both inorganic and organic cations were concentration-dependent within a concentration range of about 0.01–30 $\mu\text{eq/ml}$, at pH 7.4 and reached identical maximal values in the same batch of material. The uptakes were strongly dependent on pH, with a complete breakdown of the cation binding capacity on lowering the pH from 7–8 to 4–5. The uptake capacity at 7–8 corresponded fairly well to the number of weakly acid groups in the granule material, as indicated by the HCl consumption on titration of the granule material from pH 7 to pH 4. Taken together the observations agree with the assumption that the uptake of cations by the granule material depends on the presence of weak anionic groups, probably carboxyls. The demonstration that the uptake curves for all the cations studied fit the Rothmund-Kornfeld equation corroborates the idea that the granule material behaves as a cation exchanger. The identical affinities of the monoamines (PhEA, TA, NA, A) strongly suggest that the binding forces mainly emanate from the ionized amino groups on the side chain of the amines, the hydroxyl groups in the aromatic ring or in the side chain playing only an insignificant role.

The localization of the ionic binding sites in the granule material is unclear. After the extensive dialysis and subsequent high speed centrifugation to which the granule material was subjected mainly chromogranins remain. Since chromogranins are unusually acid proteins, they will contain considerable numbers of free carboxyl groups capable of binding cations. However to what extent anionic binding sites may also originate from solubilized granule membrane constituents, mucopolysaccharides, glycoproteins etc. remains to be studied.

It is notable that the observed cation uptake capacity corresponds to about 20–30% of the normal CA content of the bovine adrenal medullary granules, i.e. about the same percentage of the CA store which according to Hillarp (1960) could not be bound to granule ATP. However until the localization of the cation binding sites is established the biological implications of the present observations are obscure. It might be appropriate to recall our previous observations that the matrix of the basophil mast cell granules has the properties of a weak cation exchanger and that the storage and release of histamine in mast cells can be explained as cation exchange processes. Whether or not similar cation exchange mechanisms play a role in the storage and release of the catecholamines in the adrenal medulla remains to be seen. Similarly the physiological importance of the observed cation exchanger properties of material obtained from neuronal adrenergic vesicle preparations (to be published) is not yet clear.

As will be described in a later publication, the medullary granule material behaves *in vitro* like a two-compartment store for biogenic amines. With higher concentrations of aromatic amines than those described in the present paper (50–300 $\mu\text{eq/ml}$) increasing amounts (proportions) of the amines were taken up. Also in this respect the medullary granule material shows similar biogenic amine storing properties to those shown for a protamine heparin complex (PHC) by Uvnäs and Åborg (1976) and for mast cell granules by Uvnäs and Åborg (to be published). The functional implications of these observations will be dealt with in a coming publication.

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Contractility, Muscle Mass and Agonist Sensitivity of Isolated Portal Veins from Normo- and Hypertensive Rats

By

MORLEY C. SUTTER and BENGT LJUNG

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Abstract

SUTTER M. C. and B. LJUNG: *Contractility, muscle mass and agonist sensitivity of isolated portal veins from normo- and hypertensive rats*. Acta physiol scand. 1977 99 484-49

Properties of the longitudinal smooth muscle of portal veins from normotensive Wistar rats, adult (NCR) and young (NCR_y), spontaneously hypertensive Okamoto rats, adult (SHR) and young (SHR_y), and adult Wistar rats with renal hypertension (RHR) were studied *in vitro* and histologically. Some aortic strips from SHR and SHR_y were compared with controls. In response to noradrenaline (NA) and acetylcholine (ACh) greater maximum force was developed by veins from all hypertensive groups than by those from control rats. Cross-sectional area of the longitudinal muscle of veins from SHR but not SHR_y nor RHR was greater than control. Maximum stress in response to agonists was greater in both SHR and RHR than NCR. ED₅₀-values for NA and ACh were lower in portal veins from SHR than NCR but not from RHR nor SHR compared to controls. Denervation did not abolish any of the differences between SHR and NCR. Aortic strips from SHR developed less maximum force to NA and ED₅₀ was greater than those from NCR. In contrast to the findings in portal veins, low levels of external Ca²⁺ revealed altered calcium handling in veins from SHR compared to controls.

It is concluded that portal veins from hypertensive rats are functionally different from those of normotensive rats and differ in SHR compared to RHR. It is suggested that the altered functional properties of portal vein, but not of aorta, in several respects resemble those of renal resistance vessels. The implications of these findings are discussed in terms of mechanisms of hypertension in these animal models.

The spontaneously hypertensive rat (SHR) of the Okamoto strain is a much-studied model of essential hypertension. The pathogenic mechanism of this type of hypertension has been suggested to be hypertrophy of the vascular smooth muscle which is triggered by intermittent rises in arterial pressure which can be ascribed to sympathetic hyperreactivity (Folkow *et al* 1973, Hallböök 1975). Along with the development of the structural changes the hypertension is becoming increasingly sustained and also accentuated by a positive feed-back relationship between functionally induced pressure rises and the hypertrophy of the pre-capillary resistance vessels. Finch and Haeusler (1974) however have presented evidence for the existence of altered excitation-contraction coupling in the perfused mesenteric artery

paration of these animals. On the other hand, Muhany and Halpern (1976) have shown a correlation between the increased maximum force and the vascular wall thickness in sized small mesenteric arteries of SHR as compared to normotensive Wistar Kyoto rats. The question of the etiology of the vascular alterations in hypertension is not settled, in fact with hypertrophy and enhanced contractility may be involved.

There have been many studies of isolated blood vessels from hypertensive animals. Usually the arteries are used and the question arises as to how closely they functionally resemble resistance vessels. Furthermore, it is always uncertain whether any differences observed in vessels from hypertensive animals are a consequence or a cause of the high arterial pressure. Recently analyses of pressure responses to noradrenaline (NA) of consecutive segments of the vascular bed of isolated rat hindquarters perfused with solutions containing low or small amounts of calcium have indicated that contraction of the peripheral resistance vessels is much more dependent on extra-cellular calcium than is contraction in more proximal vessels (Folkow *et al.* 1976). The mesenteric portal vein is also highly dependent on extra-cellular calcium for both its myogenic spontaneous activity and agonist-induced contractions (for ref. see Collins, Sutter and Teiser 1972). Contractions of aortic strips in response to various agonists are much less dependent on external calcium (Hudgins and Veis 1968, Devine, Somlyo and Somlyo 1972). In this and other regards, the peripheral resistance vessels seem much more analogous to the mesenteric-portal vein *in vitro* than they are to aortic strips (cf. Rhodes and Sutter 1971, Folkow *et al.* 1977, Ljung 1977).

The present expts. were designed to study the rat portal vein, a vessel which is not exposed directly to the elevated systemic arterial pressure, in normo- and hypertensive rats and in a few cases to compare it to aortic strips from the same animals. The results indicate that portal veins from hypertensive rats differ from those of normotensive rats and that portal veins differ in one type of hypertension to another.

Methods

Animals

Adult male spontaneously hypertensive rats of the Okamoto strain (SHR) were approximately 11 months of age, weighed from 250-300 g and had mean arterial blood pressures (B.P. measured in the tail artery after recovery from light ether anaesthesia) of at least 135 mmHg. The young male SHR were 6-8 weeks of age, weighed 135-155 g and had mean arterial B.P. of 105-115 mmHg. Normotensive control rats (NCR) were male Wistar rats matched by weight. The adult NCR's had mean B.P. of 105-120 mmHg and the young NCR (NCR_y) had mean B.P. of 95-105 mmHg. Renal hypertensive rats (RHR) were adult male Wistar rats made hypertensive by partial occlusion of the left renal artery by silver clip (Laudgra 1974). They were used 2 months after clipping and had B.P. of 150-210 mmHg. Control animals were weight matched male Wistar rats (NCR). All animals were delivered by Møllegaard Hansen Avlslaboratorier Denmark. They were fed standard diet (Aurkoven) and given drinking water *ad lib*.

Open-bed experiments

The rats were killed by blow on the head. The portal vein was dissected free of adherent tissue, moved along its length to form a strip, and tied at both ends with silk. The vein was then excised, mounted in an organ bath and attached to Grass FT 03 force-displacement transducer under 5 mN passive force. One control vein and one vein from hypertensive animal were always mounted in the same organ bath. Force developed by the vein is recorded on Grass Model 7 polygraph and the force signal was electronically integrated over 1 min periods on separate channel.

In few expts. helical strips were prepared from the thoracic aorta and mounted under 10 mN passive force for recording of responses as with the portal vein.

Contractility, Muscle Mass and Agonist Sensitivity of Isolated Portal Veins from Normo- and Hypertensive Rats

By

MORLEY C. SUTTER and BENGT LJUNG

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Abstract

SUTTER M C and B LJUNG *Contractility, muscle mass and agonist sensitivity of isolated portal veins from normo- and hypertensive rats* Acta physiol. scand 1977 99 484-495

Properties of the longitudinal smooth muscle of portal veins from normotensive Wistar rats, old (NCR) and young (NCR_y) spontaneously hypertensive Okamoto rats, adult (SHR) and young (SHR_y) and Wistar rats with renal hypertension (RHR) were studied in vitro and histologically. Some aortic strips from SHR and SHR_y were compared with controls. In response to noradrenaline (NA) and acetylcholine (ACh) greater maximum force was developed by veins from all hypertensive groups than by those from control rats. Cross-sectional area of the longitudinal muscle of veins from SHR but not SHR_y nor RHR was greater than control. Maximum stress in response to agonist was greater in both SHR and RHR than NCR. ED₅₀-values for NA and ACh were lower in portal veins from SHR than NCR but not from RHR nor SHR_y compared to controls. Denervation did not abolish any of the differences between SHR and NCR. Aortic strips from SHR developed less maximum force to NA and ED₅₀ was greater than those from NCR, i.e. opposite to the findings in portal veins. Low levels of external Ca²⁺ reveal altered calcium handling in veins from SHR compared to controls.

It is concluded that portal veins from hypertensive rats are functionally different from those of normotensive rats and differ in SHR compared to RHR. It is suggested that the altered functional properties of portal veins, but not of aorta, in several respects resemble those of arterial resistance vessels. The implications of these findings are discussed in terms of mechanisms of hypertension in these animal models.

The spontaneously hypertensive rat (SHR) of the Okamoto strain is a much-studied model of essential hypertension. The pathogenic mechanism of this type of hypertension has been suggested to be hypertrophy of the vascular smooth muscle, which is triggered by intermittent rises in arterial pressure which can be ascribed to sympathetic hyperreactivity (Folkow *et al.* 1973; Hallböök 1975). Along with the development of the structural changes the hypertension is becoming increasingly sustained and also accentuated by a positive feedback relationship between functionally induced pressure rises and the hypertrophy of the precapillary resistance vessels. Finch and Haeusler (1974), however, have presented evidence for the existence of altered excitation-contraction coupling in the perfused mesenteric artery.

On sabbatical leave from Department of Pharmacology University of British Columbia, Vancouver V6T 1W5 Canada.

TABLE 1. Maximum peak force, and mean force levels obtained by integration, during spontaneous activity of the portal vein from normotensive and hypertensive rats and maximum force developed in response to noradrenaline or acetylcholine as determined from complete concentration-response curves (mean \pm S.E. Number of observations (n) given in brackets. Maximum responses were not always sought in each tissue hence the sum of () of the last two columns does not always equal the () of the first column. For definitions of groups see Methods indicates $p < 0.05$

Group	Spontaneous activity		Maximum response to agonist (mN)	
	Maximum force (mN)	Mean force (mN)	Noradrenaline	Acetylcholine
NCR	5.8 ± 0.4 (26)	3.4 ± 0.3 (19)	12.0 ± 1.0 (11)	13.8 ± 1.4 (6)
SHR	9.8 ± 0.5 (14)	3.7 ± 0.4 (11)	22.3 ± 0.5 (5)	21.2 ± 0.9 (4)
RHR	8.0 ± 0.5 (6)	5.1 ± 0.6 (6)	16.8 ± 0.5 (6)	23.9 ± 8.1 (3)
SHR _y	6.0 ± 0.6 (9)	4.3 ± 0.7 (9)	13.2 ± 0.8 (7)	
RHR _y	6.2 ± 0.6 (9)	3.9 ± 0.5 (9)	15.7 ± 0.7 (7)	
SHR _{ad}	5.6 ± 1.7 (5)	3.3 ± 1.1 (5)	9.7 ± 1.7 (3)	
RHR _{ad}	10.5 ± 1.0 (9)	6.4 ± 0.9 (9)	20.6 ± 1.8 (9)	
SHR _{ad} + yz	6.8 ± 0.8 (4)	5.0 ± 1.1 (4)	21.0 ± 0.9 (4)	
RHR _{ad} + yz	6.0 ± 0.8 (4)	4.6 ± 0.5 (4)	16.5 ± 2.0 (4)	
SHR _{ad} + yz + pz	6.5 ± 1.0 (4)	4.4 ± 0.7 (4)	20.4 ± 1.2 (4)	

concentration and with no agonist added. Between contractions complete relaxation to the baseline of passive force occurred. Low concentrations of NA and ACh induced oscillatory responses which were characterized by increased rate and amplitude of the phasic activity. With increasing concentration of agonists the phasic contractions tended to fuse and a maximum responses—smooth, maintained level of active force was regularly recorded. The peak spontaneous force expressed as a percentage of the maximum force induced by agonists was comparable in the different groups of rats and was found to range between 40 and 50 per cent.

Table 1 summarizes the results obtained when spontaneous contraction and maximum responses to agonists were quantitated in absolute terms. The spontaneous activity was higher in SHR and RHR veins compared to NCR veins both with regard to peak force of isolated contractions and to mean force obtained by integration. Similarly the maximum force developed in response to NA or ACh was greater in SHR compared to NCR rats. Symphectomy produced no reduction of force developed during spontaneous contractions of SHR veins nor in their response to NA. Protriptyline seemed to reduce the peak force of individual spontaneous contractions but did not significantly alter mean force developed spontaneously in veins from SHR nor to affect maximum force developed in response to NA. As in young rats, veins from SHR_y and NCR_y were similar in terms of force developed during spontaneous contractions but the maximum force in response to NA was larger in SHR_y veins.

In addition to the studies on isolated portal vein preparations the maximum force developed in thoracic aortic strips in response to NA was compared for 5 NCR and 5 SHR rats. Strips from NCR contracted more strongly than those from SHR, 15.3 ± 2.1 and 12.6 ± 2.1 mN (mean \pm S.E.), respectively ($p = 0.002$ by paired *t*-test), and this is opposite to the findings in the portal vein. Maximum contractions produced by NA were also greater in

The fluid bathing the tissues was of the following composition in mM: NaCl 122, KCl 4.73, NaHCO₃ 15.5, KH₂PO₄ 1.19, MgCl₂ 1.19, glucose 11.5, CaNa₂-versenate 0.026. A CaCl₂ concentration of 2.5 mM was designated 'normal'. The solution was continuously bubbled with 4% CO₂ in O₂ and kept at 38°C.

The tissues were allowed to stabilize for at least 1 h before responses were induced. Agonists were added in volumes of 1 per cent of the bath fluid. Cumulative concentration-effect curves were made by proceeding from lowest to highest concentration of agonist and allowing each concentration to be in contact with the tissue for 1 min. The average integrated force developed during the 1 min exposure period minus the mean spontaneous force developed during the preceding 2 min control period was then used as the effect of the drug. These values were entered into a computer program which calculated the log ED₅₀ value for each concentration-effect curve by a least-squares fit to a true hyperbolic function.

In expts. where calcium concentration was altered the protocol was as follows: a concentration-effect curve was first obtained in normal 2.5 mM calcium concentration and then the bath solution was changed into one with a different calcium concentration. After 30 min in the altered Ca²⁺ solution another NA concentration-effect curve was obtained. The sequence of calcium concentrations was 3 mM (normal), 0.0, 0.1, 0.2, 0.4, 0.8 mM of added calcium and then back to normal calcium.

The following agonists were used: noradrenaline hydrochloride, (*l*-arterrenol, Sigma) and acetylcholine chloride (Merck). All concentrations are expressed in terms of the base. Noradrenaline (NA) solutions are made up daily from a stock solution kept refrigerated and acidified with 10 mM HCl. The stock solution of acetylcholine (ACh) was kept frozen until the day of its use when it was diluted appropriately. In all cases agonists were diluted *in saline*.

In expts. where ACh was the agonist, phentolamine, 0.3 µM was constantly present in the bath fluid to inhibit any denervated response due to ACh-induced release of catecholamines from the tissue. In a few expts. on denervated portal veins (SIR_{den-veg}) and on veins from sham-operated animals (SIR_{sham-veg}) propranolol 0.1 µM was present in the bathing fluid.

Surgical procedure

The portal vein of 20 rats was denervated as described previously (Johansson *et al.* 1970) and the vein used 10–14 days later. Controls (SIR_{sham}) were sham-operated on the same day as experimental animals. The criteria for successful sympathectomy was response to a 1 min transmural stimulation with square wave pulses (8–16 Hz, 15 V, 0.8 ms) which was 10% or less of the response in a non-denervated vein or of the maximum response to NA.

Histology

At the end of the organ bath expt. the bath was lowered and a beaker filled with Bouin's solution brought into position and the tissues were fixed overnight (16 h) while attached to the force transducer at the length which prevailed during the expt. The portal veins were then transferred to individual vials filled with Bouin's fluid and stored until processed further 2–3 weeks later. The fixed tissues were dehydrated in alcohol, cleared in xylene, embedded and sectioned (5 µm) perpendicularly to the longitudinal axis of the vessel. Sections corresponding to the mid-point along the length of the portal vein preparation were selected and stained with Weigert's hematoxylin and van Gieson methods.

The sections were photographed in Zeiss photo microscope at 400 magnification. A series of code prints and a simultaneously obtained photograph of a calibration scale were provided to the examiner. He traced the cross-sectional area of the longitudinal muscle layer of the portal vein on heavy duty transparent paper. Likewise a square corresponding to 1 mm² was prepared. The tracings were cut, weighed, the code was deciphered and the cross-sectional areas, expressed in mm² were calculated for the effector tissue.

Statistics

Results in control and experimental groups were compared using Student's *t* test for group data and occasionally where appropriate, for paired data. A *p*-value of less than 0.05 was accepted as showing significant difference.

Results

Contractile force

In all rat portal veins studied, spontaneous phasic contractions at a rate of approximately 2–6 contractions per min were recorded in the control situation, *i.e.* at normal (2.5 mM)

erized veins from NCR due to elimination of any trophic influence exerted by the motor nerves. Surgery on the portal vein led to an increased variability of cross-sectional area of the longitudinal muscle layer from one vein to another and this is indicated by the large standard errors seen in these groups. No statistical difference could be shown between any of these groups. Denervation of veins from NCR was associated with a striking increase in the average area of their longitudinal muscle layer. The purpose of these experiments, however, was to compare denervated SHR and NCR, hence the lack of an appropriate sham-operated NCR group. Thus and the small numbers of observations precludes a definite conclusion as to this apparent effect.

The right-hand panel of Fig. 1 shows the values for maximum stress (force per unit area) obtained in the various groups of portal veins in response to NA. Compared to NCR, both DR and RHR veins develop much greater stress. Thus, the increased maximum contractile force of SHR and RHR in comparison with NCR shown in Table I cannot be explained by increased effector mass alone but reflects enhanced contractility of the smooth muscle.

Smooth muscle sensitivity and relative responses to agonists in low concentrations of Ca^{2+}

Smooth muscle sensitivity to NA or ACh expressed as mean log ED_{50} values was determined from cumulative concentration-response relationships (see "Methods"). Fig. 2 illustrates mean log ED_{50} values obtained at normal (2.5 mM) and at several low Ca^{2+} concentrations. In SHR portal vein displayed greater sensitivity to NA (Fig. 2 left panel) and to ACh (Fig. 2 right panel) at practically all Ca^{2+} concentrations tested as seen from the log ED_{50} values which range significantly below those of the NCR portal veins at most Ca^{2+} levels. In contrast, the aortic strips from the same groups of animals (Fig. 3 left panel) showed that the smooth muscle of this elastic artery from SHR was less sensitive to NA than that from NCR over the same range of Ca^{2+} concentrations. In portal vein preparations from SHR and NCR, the mean NA log ED_{50} values were not significantly different except at a Ca^{2+} concentration of 0.2 mM which was the lowest one tested (5.28 ± 0.04 and 5.11 ± 0.05 (M) \pm S.E., $n = 6$, respectively) and during the second exposure to normal 2.5 mM calcium concentration (6.28 ± 0.08 and 6.02 ± 0.05 (M), respectively). Furthermore, the ED_{50} values for NA were entirely comparable in RHR and NCR portal veins at all Ca^{2+} levels.

Another way of examining the function of the portal vein from normotensive and hypertensive rats in graded low concentrations of Ca^{2+} is illustrated in Fig. 3. The peak contraction amplitude of the spontaneous activity (SA) or the mean force developed in response to each concentration of NA during the initial concentration-effect curve in normal Ca^{2+} concentration have been set at 100 per cent. The SA amplitude and the individual responses to NA at the several low Ca^{2+} concentrations have then been expressed as percentages of their respective control values. It is apparent that the spontaneous activity which is myogenic in origin, is practically abolished in all preparations at Ca^{2+} concentrations of 0.8 mM or less. Responses to exogenous NA, on the other hand, show a NA concentration dependent increase in the percentage remaining in low calcium. It may be noted that the maximum response at 0.8 mM and at 2.5 mM often exceeded 100 per cent. This is probably due to the fact that the maximum responses of both SHR and NCR tended to grow in the course of the

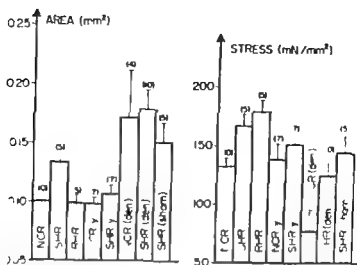


Fig. 1 *Left diagram.* Cross-sectional area of longitudinal media layer of portal veins from normotensive and several types of hypertensive rats. *Right diagram.* Maximum stress in response to noradrenaline calculated from maximum force values (mN) given in Table I and cross-sectional area (left). Mean \pm S.E. Number of observations with parentheses. * indicates significant difference ($p < 0.05$) from appropriate control group.

aortic strips from 3 NCR_y compared to strips from 3 SHR_y (6.8 ± 1.1 and 3.1 ± 0.4 mN mean \pm S.E., $p = 0.03$).

Cross-sectional area and maximum stress

The maximum force developed in response to NA was related to the cross-sectional area of the longitudinal muscle of the portal vein as determined by light microscopy in order to obtain a measure of maximum stress (mN/mm²) in the individual tissues.

Preliminary studies showed that within the segment used the longitudinal muscle layer of the portal vein is reasonably uniform in as much as the maximum force developed was not altered by shortening the tissue. This was demonstrated by suspending a vein in an organ bath and measuring the maximum force developed in response to NA, $10 \mu\text{M}$. Then the vein was tied at the mid-point, cut and resuspended at the same passive force so that the length of tissue exerting force was reduced to one half the previous length. The length of tissue could be reduced several times in this manner without altering maximum force induced by NA. Therefore it seemed reasonable to measure cross-sectional area of longitudinal muscle at the mid point of each tissue and relate it to the maximum force developed by the portal veins from NCR, SHR, RHR, NCR_y, SHR_y, denervated SHR, denervated NCR and sham-operated SHR.

The results of these studies are shown in Fig. 1. In the left-hand panel it can be seen that the area of the longitudinal muscle layer of portal veins from SHR was greater than that of NCR controls, whereas veins from RHR were not different from NCR in this regard. The average area of the longitudinal muscle layer in veins from SHR_y was slightly greater than that of NCR_y, but was not statistically different.

Denervated veins from SHR were examined to see whether chronic sympathectomy would alter the mass of effector tissue compared to sham-operated veins from SHR and

and veins from NCR due to elimination of any trophic influence exerted by the splanchnic nerves. Surgery on the portal vein led to an increased variability of cross-sectional area of the longitudinal muscle layer from one vein to another and this is indicated by the standard errors seen in these groups. No statistical difference could be shown between these groups. Denervation of veins from NCR was associated with a striking increase in average area of their longitudinal muscle layer. The purpose of these experiments, however, is to compare denervated SHR and NCR, hence the lack of an appropriate sham-operated group. That and the small numbers of observations precludes a definite conclusion as to this apparent effect.

The right-hand panel of Fig. 1 shows the values for maximum stress (force per unit area) developed in the various groups of portal veins in response to NA. Compared to NCR, both SHR and RHR veins develop much greater stress. Thus, the increased maximum contractile force of SHR and RHR in comparison with NCR shown in Table 1 cannot be explained by increased effector tissue mass alone but reflects enhanced contractility of the smooth muscle.

Smooth muscle sensitivity and relative responses to agonists in low concentrations of Ca^{2+}
 Effector sensitivity to NA or ACh expressed as mean log ED_{50} values was determined from cumulative concentration-response relationships (see Methods¹⁷). Fig. 2 illustrates the log ED_{50} values obtained at normal (2.5 mM) and at several low Ca^{2+} concentrations. RHR portal vein displayed greater sensitivity to NA (Fig. 2 left panel) and to ACh (right panel) at practically all Ca^{2+} concentrations tested as seen from the log ED_{50} values which range significantly below those of the NCR portal veins at most Ca^{2+} levels. In contrast, the aortic strips from the same groups of animals (Fig. 2 left panel) showed that smooth muscle of this elastic artery from SHR was less sensitive to NA than that from NCR over the same range of Ca^{2+} concentrations. In portal vein preparations from SHR, NCR, the mean NA log ED_{50} values were not significantly different except at a Ca^{2+} concentration of 0.1 mM which was the lowest one tested (5.28 ± 0.04 and 5.11 ± 0.05 (M) \pm S.E., $n = 6$, respectively) and during the second exposure to normal 2.5 mM calcium concentration (6.28 ± 0.08 and 6.02 ± 0.05 (M), respectively). Furthermore, the ED_{50} values for NA were entirely comparable in RHR and NCR portal veins at all Ca^{2+} levels. Another way of examining the function of the portal vein from normotensive and hypertensive rats is graded low concentrations of Ca^{2+} is illustrated in Fig. 3. The peak contraction level of the spontaneous activity (SA) or the mean force developed in response to each contraction of NA during the initial concentration-effect curve in normal Ca^{2+} concentrations have been set at 100 per cent. The SA amplitude and the individual responses to NA at several low Ca^{2+} concentrations have then been expressed as percentages of their active control values. It is apparent that the spontaneous activity which is myogenic in nature, is practically abolished in all preparations at Ca^{2+} concentrations of 0.8 mM or less. Responses to exogenous NA, on the other hand, show a NA concentration dependent increase in the percentage remaining in low calcium. It may be noted that the maximum response at 0.8 mM and at 2.5 mM often exceeded 100 per cent. This is probably due to the fact that the maximum responses of both SHR and NCR tended to grow in the course of the

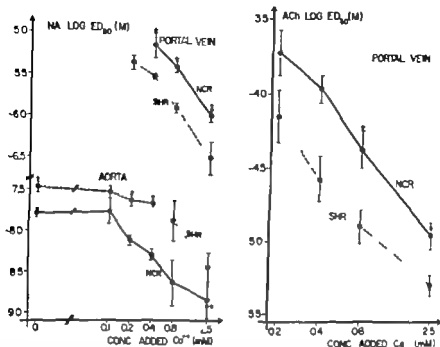


Fig. 2. Mean log ED₅₀ values (\pm S.E., $n=5$) to norepinephrine (NA) determined in portal vein preparations and aortic strips (A) and mean log ED₅₀ values (\pm S.E.) to acetylcholine (ACh) determined in portal vein preparations (B) from normotensive (NCR) and spontaneously hypertensive rats (SHR) at portal Ca concentration (2.5 mM) and at several low Ca concentrations. Square symbols illustrate mean log ED values determined in 2.5 mM calcium in low rats end of experiment (See Methods for protocol). $p < 0.05$. Note that SHR portal vein is more sensitive to NA than NCR portal vein at all Ca levels here the opposite applies to the aortas and that portal vein from SHR is a significantly more sensitive than NCR ACh at most Ca concentration.

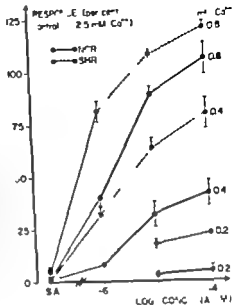
experiment as reflected by the observation that the final maximum response to NA in the last (second) exposure in 2.5 mM calcium was almost always greater than the maximum NCR response at the same Ca concentration at the beginning of the experiment.

In the adult SHR the residual responses to NA in low Ca concentrations were greater than those of NCR whereas no such difference occurred between SHR₇ and NCR. When ACh was the agonist the relative remaining response also were of comparable magnitude in SHR and NCR.

Aortic strips from SHR were found to retain a smaller percentage of their control NA response than did NCR strips at all Ca concentrations studied. Actually the per cent values at 0.4 and 0.8 mM Ca for responses of the NCR to NA at levels ranging from 1 nM to 10 μ M all exceeded 50 per cent and did not show much variation with agonist concentration. Thus the aortic and portal vein smooth muscles seem to handle calcium quite differently and the dependence on external Ca for responses to NA is altered in opposite directions in these two tissues from SHR compared to NCR.

Discussion

Our results show that portal veins from hypertensive rats are functionally different from those of matched normotensive Wistar rats which served as controls. In all hypertensive



Contractile responses of portal vein from normotensive (NCR solid line) and from spontaneously hypertensive rats (SHR interrupted line) to graded concentrations of acetylcholine (ACh) at graded concentrations of acetylcholine (ACh). Responses expressed as % of control responses to corresponding concentrations determined during initial dose-response curve in 2.5 mM Ca^{2+} concentration. Mean \pm S.E. Note that spontaneous activity decreases and that the residual responses are maintained in dose dependent manner.

As for SHR, SHR₁, and RHR, the portal vein preparation had increased maximum responses to agonists compared to controls. The aortic strips from SHR, however, showed a decreased maximum, when compared to strips from NCR. Several workers have previously reported the decreased maximum responses to agonists in aortic strips from SHR (for *et al* 1969, Cline-Schmidt *et al* 1970, Shibata, Kurihara and Kuchii 1973). Green and Bohr (1975) reported findings similar to ours in portal veins from SHR but the responses of portal veins from SHR₁ and RHR have not been described previously to our knowledge. Cline-Schmidt *et al* (1970) demonstrated that the maximum response of aortic strips varied from one strain of rat to the other and pointed out the need for proper controls. The Wistar strain used in the present experiments represents the most appropriate normotensive animal for comparisons to SHR which is available to us at present. Nevertheless, there is an element of uncertainty present when quantitative comparisons have to be made between animals of different families. However the fact that RHR portal veins show increased maximum responses to agonists and increased spontaneous activity compared to SHR—which were of identical strain—lends support to the tenet that portal veins from hypertensive animals are indeed different from those of control animals. The observation that aortic strips had decreased maxima while the portal vein from the same SHR had increased maxima as compared to control indicates interesting regional variation in the properties of vascular smooth muscle of the hypertensive animal.

The increased maximum response in SHR portal veins is associated with both increased tension of the longitudinal muscle and increased stress (force per unit area) whereas aortic strips from RHR develop increased stress in response to agonists but are not hyperphasic. Since the portal vein is not directly exposed to the increased arterial blood pressure, it suggests that local hypertension *per se* is not responsible for the altered properties of

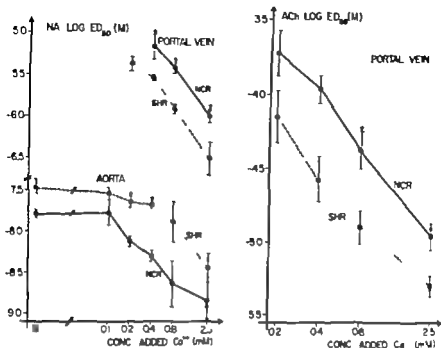


Fig. 2. Mean log ED₅₀ values (\pm S.E., $n=5$) to noradrenaline (NA) determined in portal vein preparations and aortic strips (A) and mean log ED₅₀ values (\pm S.E.) to acetylcholine (ACh) determined in portal vein preparations (B) from normotensive (NCR) and spontaneously hypertensive rat (SHR) at normal Ca²⁺ concentration (2.5 mM) and at several low Ca²⁺ concentrations. Squares symbols illustrate mean log ED₅₀ values determined 2.5 mM calcium towards end of experiment (See Methods for protocol). * denotes $p < 0.05$. Note that SHR portal vein is more sensitive to NA than NCR portal vein at all Ca²⁺ levels whereas the opposite applies to the aortas and that portal vein from SHR is significantly more sensitive than NCR to ACh at most Ca²⁺ concentrations.

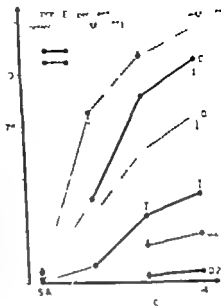
experiment as reflected by the observation that the final maximum response to NA in the last (second) exposure in 2.5 mM calcium was almost always greater than the maximum NA response at the same Ca²⁺ concentration at the beginning of the experiment.

In the adult SHR the residual responses to NA in low Ca²⁺ concentrations were greater than those of NCR whereas no such difference occurred between SHR and NCR, nor between SHR and NCR. When ACh was the agonist the relative remaining responses were also of comparable magnitude in SHR and NCR.

Aortic strips from SHR were found to retain a smaller percentage of their control NA response than did NCR strips at all Ca²⁺ concentrations studied. Actually the per cent values at 0.4 and 0.8 mM Ca²⁺ for responses of the NCR to NA at levels ranging from 1 nM to 10 μ M all exceeded 50 per cent and did not show much variation with agonist concentration. Thus the aortic and portal vein smooth muscles seem to handle calcium quite differently and the dependence on external Ca²⁺ for responses to NA is altered in opposite directions in these two tissues from SHR compared to NCR.

Discussion

Our results show that portal veins from hypertensive rats are functionally different from those of matched normotensive Wistar rats which served as controls. In all hypertensive



1. Contractile responses of portal vein from normotensive rats (NCR solid line) and from spontaneously hypertensive rat (SHR interrupted line) to norepinephrine (NA) at graded concentrations of 10^{-8} M. Square symbols indicate amplitude of passive activity (SA). Responses expressed as percentage of control responses to corresponding concentrations determined during initial dose response curve at 10^{-8} M NA concentration. Mean \pm S.E. $n = 5$. Note that spontaneous activity decreases with dose and that the residual responses to NA are maintained in a dose dependent manner.

expts. 1. SHR, SHR₂, and RHR, the portal vein preparation had increased maximum responses to agonists compared to controls. The aortic strips from SHR, however, showed the opposite: decreased maxima, when compared to strips from NCR. Several workers have previously reported the decreased maximum responses to agonists in aortic strips from SHR (Pocock *et al.* 1969; Clineschmidt *et al.* 1970; Shibata, Kurahashi and Kuchii 1973). Greenfield and Boler (1975) reported findings similar to ours in portal veins from SHR but the maximum responses of portal veins from SHR₂ and RHR have not been described previously to our knowledge. Clineschmidt *et al.* (1970) demonstrated that the maximum response of aortic strips varied from one strain of rat to the other and pointed out the need for proper controls. The Wistar strain used in the present expts. represents the most appropriate nor-tensive animal for comparisons to SHR which is available to us at present. Nevertheless, there is an element of uncertainty present when quantitative comparisons have to be made between animals of different families. However, the fact that RHR portal veins show increased maximum responses to agonists and increased spontaneous activity compared to NCR—which were of identical strain—lends support to the tenet that portal veins from hypertensive animals are indeed different from those of control animals. The observation that aortic strips had decreased maxima while the portal vein from the same SHR had increased maxima as compared to control indicates interesting regional variation in the properties of vascular smooth muscle of the hypertensive animal.

The increased maximum response in SHR portal veins is associated with both increased mass of the longitudinal muscle and increased stress (force per unit area) whereas portal veins from RHR develop increased stress in response to agonists but are not hypertrophied. Since the portal vein is not directly exposed to the increased arterial blood pressure, this suggests that local hypertension *per se* is not responsible for the altered properties of

ceptors. The portal veins from RIIR did not display increased agonist sensitivity. This suggests that the mechanisms involved in the increased portal vein responsiveness in RIIR are different from those of SHIR.

Hallböök, Lundgren and Werns (1971) observed no difference in NA ED_{50} values in portal veins from SHIR and NCR. Most methodological aspects were the same in their study except for two points. We calculated mean log ED_{50} values (Fleming *et al.* 1973) from cumulative concentration-response curves whereas they used arithmetic means and non-cumulative concentration-response curves. It is interesting to note that desensitization to NA (but not ACh) occurred in our experiments. The second concentration-response curve in normal calcium concentration for the portal vein from SHIR gave a higher ED_{50} value than the first one (Fig. 7). In fact, the second ED_{50} in normal calcium in the SHIR and NCR portal vein did not differ significantly from the other. In control experiments it was found that the desensitization in the SHIR was due to the repeated exposure of the tissue to NA and not simply passage of time or exposure to altered calcium concentration (Sutter and Jung, unpublished). Thus, it did occur on repeated exposure to NA at a constant calcium concentration 2.5 mM. It did not occur when the same period of time was simply allowed to elapse between one initial and one final dose-response curve to NA without intervening exposures to NA. It is also interesting to note that the ED_{50} to NA in 0.8 mM calcium was different in RIIR compared to NCR portal veins (Fig. 8) even though this ED_{50} determination was very close to the second ED_{50} in normal calcium where no difference in ED_{50} was seen between the SHIR compared to the NCR portal vein.

Another difference among the portal veins from hypertensive rats involved their handling of calcium. Portal veins from SHIR retained their responses to NA in low calcium better than those from NCR. Veins from SHIR, and RIIR were not different from controls in this regard. Similar results have been found in perfused rat hind-quarters (Folkow *et al.* 1977 and unpublished), where SHIR were found to retain their previous responses to NA in low calcium better than controls but SHIR only minimally showed this effect and RIIR not at all. We found no consistent difference in per cent response retained in low calcium when SHIR veins were compared to NCR veins with ACh as the agonist. This may indicate variation in the effects of NA and ACh on calcium kinetics in the portal vein, a previously shown for baroreceptors based on differential blockade of the responses to NA and ACh by tetraethylammonium and Vanhoose (1975).

Finally one must ask what could cause the structure and function of a vessel like the portal vein to be altered in hypertensive rats? There have been reports of decreased contractility of femoral and mesenteric arteries in rats and in man (Overbeck 1972, Simon *et al.* 1973) in perinephritic dogs. These authors concluded that structural alterations of the vessels are involved. Simon (1976) has reported that SHIR also shows decreased venous compliance. Bevan *et al.* (1975 a, b, and 1976) have reported that veins from rabbits made hypertensive by partial constriction of the aorta proximal to the renal arteries show increased responsiveness to NA and to nerve stimulation which they attribute to an increased density of α -adrenergic receptors. The physiology of the portal vein which we used is unlike the saphenous and cephalic veins these latter workers studied, so that their

the portal vein. At present it cannot be ruled out completely that the transmural pressure of the portal vein is in fact elevated in hypertension. However the difference between RHR and SHR with regard to cross-sectional area seems to indicate that in SHR as opposed to RHR there may be a systemic factor relatively independent of pressure which produces hypertrophy. Greenberg and Bohr (1975) did not find hypertrophy in their study of portal veins from SHR but they measured cross-sectional area by dividing weight by length. This technique fails to separate out longitudinal from circular muscle and connective tissue while the histological method we used has the merit of measuring only the longitudinal muscle layer. It is this latter muscle alone which is involved in development of force in the suspended vein. It should be pointed out that light microscopy in turn, has the limitation of neither providing information about the actual muscle cell mass nor the absolute amount of contractile filaments of the transverse section of the longitudinal muscle layer. Consequently it is possible that the increased stress values, based on cross-sectional areas determined by light microscopy in portal veins from hypertensive animals may reflect increased relative mass of contractile elements.

Trophic influences from the sympathetic vasomotor fiber supply of the portal vein might be one factor which induces and maintains the hypertrophy of the SHR portal vein. Our attempt to analyze whether chronic denervation would eliminate the differences between SHR and NCR portal veins was complicated by the surgery apparently increasing the variability of response from one vein to another. It seems clear however that denervation did not reduce the increased maximum response to agonists nor did it alter the hypertrophy of portal veins from SHR. If anything denervation produced increased cross-sectional area in veins from NCR and decreased their maximum responses. It may be that a week or 10 days was insufficient time for the effects of denervation to be seen but no longer time was allowed because of this risk of re-innervation and development of postjunctional supersensitivity. It might be that denervation not only of the portal vein but also of the arterial resistance vessels of the splanchnic region caused increased transmural pressure in the portal vein. Thus the surgical procedure would not only eliminate a possible trophic influence but could cause hypertrophy of the myogenically active smooth muscle.

Clear differences both in structure and function exist among the portal veins from the several hypertensive groups of rats. The lack of hypertrophy in veins from SHR and RHR has already been mentioned. An additional difference involves the sensitivity to agonists. Portal veins from adult SHR showed lower ED_{50} values for both NA and ACh than did NCR portal veins at all calcium concentrations studied. In the portal veins from young rats the NA ED_{50} value was significantly lower in SHR compared to NCR only at 0.2 mM Ca^{2+} concentration. This suggests that the increased agonist sensitivity in SHR veins is part of an ongoing process during the development of hypertension. Thus, at the age of 6-8 weeks (SHR₇) it is demonstrable only upon provocation by the low calcium concentration. Adrenergic receptor stimulation was not involved in the determination of ACh concentration effect curves, because phentolamine, in concentrations which blocked 90 per cent of the response to a maximum dose of NA, was present in these experiments. The fact that the ED_{50} values to both NA and ACh were lower in SHR than in NCR portal veins means that the mechanisms responsible for the increased sensitivity in SHR are not limited to adrenergic

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results may have limited relevance to the present study. Taken together however all these reports indicate that vascular changes in the hypertensive state are not limited to arteries. It is entirely possible that slightly elevated transmural pressures may operate on the venous side in hypertensive states and thus induce secondary changes. Furthermore, humoral and neurogenic influences in hypertension would be expected to affect arterial and venous muscle.

Our results demonstrate that portal veins from hypertensive animals are different from veins from normotensive animals. We indicated in the Introduction that the portal vein in several functional respects resembles the true resistance vessels, for example in its basic physiology and in dependence on extracellular calcium ion concentration (Rhodes and Sutter 1971, Folkow *et al* 1977, Ljung 1977). Thus in the hypertensive state it may well be affected by generalized factors in a manner similar to resistance vessels. If this is so, our results also indicate the following: the hypertrophy seen in arteries of perfused hindquarters of SHR (Folkow *et al* 1973) would not entirely be due to high arterial pressure but may involve a systemic, perhaps genetic tendency of vascular smooth muscle to hypertrophy in these animals. Increased maximum contractility is involved in the higher arterial pressure observed in SHR and their vascular smooth muscle seems to handle calcium differently from controls. The mechanism of hypertension in RHR is different from that in SHR but again involves increased maximum responses to agonists. The latter is independent both of vascular architecture and of adrenergic receptors and hence may involve more efficient coupling between stimulus and vascular smooth muscle contraction.

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inc. oesoph. inserted into the corporeal region of the stomach. Since the were hung on their
le. gastric juice left the gastric cavity in the caecum. Gastric juice samples are collected every
nd control for their acid content with 0.01 M NaOH. Phenolphthalein is used as an in-
pl. nerves were cut in the cervical region. The distal ends were stimulated electrically (10 Hz,
5 ms duration and 10 Hz. Effect on heart rate and blood pressure is taken as evidence of
and stimulation.

which the gastric output was determined quantitatively with additional surgical procedures. One of the incisions below the diaphragm, and the spleen, are taken away. The remnant is a partial omentum composed of blood draining the stomach, but also of some diaphragmatic and pancreatic blood. It is called the gastric blood. The portal vein was ligated close to the liver and the H₂O end of the splanchnic vein is cannulated. Thus the gastric blood is converted back into one of the splanchnic blood chamber unit, allowing continuous recording of the rate of blood flow and then led into the ca. through the portal vein. All blood samples were collected from the gastric blood and gastric determinations were made continuously (Nelson 1975). The gastric output rate is calculated as the product of recording and rate of gastric plasma flow. The experiments in which gastric acid secretion or flow was studied, were differently designed.

† If dropped and mathematics are equally induced gastric acid secretion.

Animals were performed sub 2 to 10 Hz for 1 to 1.5. A response (0.1-0.3 mg/kg or more) was given, then the secretory response seemed to have reached plate (by 40 min or more) as began 1 each until the secretory output following to decrease 7.5 min was calculated for the last 15 min period before and the first 15 min period after adjustment of the drug. The amount of HCl secreted per 15 min after the drug had been given, was then expressed as the amount secreted during the first 15 min.

effect of soropur and methanolic on orally induced gastric release

stimulations are performed at 2 and 5 Hz for 5 or 10 min. T identical stimulations are performed with Atropine 0.1 mg/kg or methacholine 20 mg/kg given 15 min after the first and 10 min before the second stimulation. The gastric output following the two stimulations was calculated as the difference between the two stimulations as expressed in per cent of the amount of gastric retained by stimulation.

Following drugs are used Atropine sulphate (Atropine, ACO), Metharnade (Smith, Kline and Beecham Ltd).

Results

the of HCl secretion induced by vagal stimulation with 2-10 Hz was clearly reduced by doses of 0.1-0.3 mg/kg (n = 6) (Fig. 1). The gastric juice collected between 15-30 min after atropinization averaged ~40% and ~30% of the amounts secreted during the control period (Table 1a).

the effect of methamide on vagally induced gastric secretion was tested in doses of 4 and 20 mg/kg (n = 3). The rate of gastric juice secretion was ~ 30 and 10 respectively in the first and second 15-min period following administration of methamide (Fig. 2, 1b).

On the other hand, the gastrin release response to vagal stimulation at 2 and 5 Hz was not affected by atropine in doses varying from 0.2–2 mg/kg (n = 10, Table II b, Fig. 3). Administration of atropine did in fact lead to a slight increase in the vagally induced gastrin output (150%). The corresponding value, when no drugs were given was ~110% (n = 5, Table II a). Methamidate 20 mg/kg did not reduce the gastrin output following vagal stimulation (n = 4) (Fig. 4).

Effect of Atropine and Methamidate on Vagally Induced Gastric Acid Secretion and Gastrin Release in Anesthetized Cats

By

KERSTIN UVNÄS-WALLENSTEN and HARRIET ANDERSSON

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Abstract

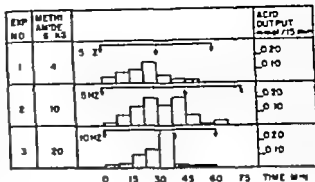
UVNÄS-WALLENSTEN, K. and H. ANDERSSON *Effect of atropine and methamidate on vagally induced gastric acid secretion and gastrin release in anesthetized cats* Acta physiol. scand. 1977 99 496-502.

Gastrin release and HCl secretion was induced by electrical vagal stimulation in anesthetized cats. Both atropine (0.1-0.3 mg/kg) and methamidate (4-70 mg/kg) inhibited the HCl secretion (by ~60 and ~80 %). On the other hand, neither atropine (0.1-2 mg/kg) nor methamidate (20 mg/kg) inhibited the vagally induced gastrin release. Possible explanations of the atropine resistance of the vagal gastrin release mechanism are discussed.

In the cat, electrical vagal stimulation induced a release of gastrin into the gastric venous outflow and also initiated a secretion of gastric juice (Uvnäs *et al.* 1975 and Uvnäs Wallensten *et al.* 1976). However, while the secretion of gastric juice continued as long as the stimulations were performed, the antral gastrin output declined after an initial peak and basal gastrin levels were reached again within 15-20 min. This difference between the two vagal responses indicates, that the nervous effector mechanisms behind these effects may be dissimilar. On continuous vagal stimulation with less than 2-3 000 impulses the gastrin output per impulse was constant. This feature of the gastrin release mechanism makes the cat preparation well suited for quantitative testing of the influence of substances on vagally induced gastrin release. The present study was performed to investigate to which extent atropine and methamidate antagonize gastric acid secretion induced by electrical vagal stimulation directly at the parietal cell level or via a diminished release of gastrin.

Methods

The experiments were performed on cats anesthetized by chloralose (50 mg/kg) and urethane (100 mg/kg). The cats weighed between 2-4 kg and had been deprived of food for 18 h. Blood pressure, pulse rate and temperature were continuously recorded and the cats were artificially ventilated. The cat were provided



3 expts. demonstrating the dose effect of methamide on acid induced HCl secretion. 10 stimulations are performed 5 and 10 Hz. Methamide 8 mg/kg is administered in the HCl secretion had died plateau level (see also Table).

Fig. 1 and 11 b Two successive (10) stimulations are performed 2 or 5 Hz for 10 min in 5 Hz (11 a). In 10 other cats atropine or doses ranging from 0.2 mg/kg are given 10 min before the second stimulation (2 or 5 Hz) (Table 11 b). The gastric output following the second stimulation was expressed as per cent of the preceding control stimulation. The interstimulatory period was 20-30 min.

Fig. 11

Expt	Frequency Hz	Gastric release induced by the second stimulation as per cent of the control stimulation (100%)
1	5	115
2	5	95
3	5	103
4	5	119
5	5	115

Mean: 106 per cent.

Fig. 11 b

Expt	Frequency Hz	Atropine mg/kg	Gastric release induced by the second stimulation as per cent of control stimulation (100%)
1	2	0.2	72
2	2	0.3	97
3	2	0.3	440
4	2	0.6	185
5	2	2.0	103
6	5	0.25	152
7	5	0.25	83
8	5	0.5	134
9	5	1.0	158
10	5	2.0	103

Mean: 153 per cent.

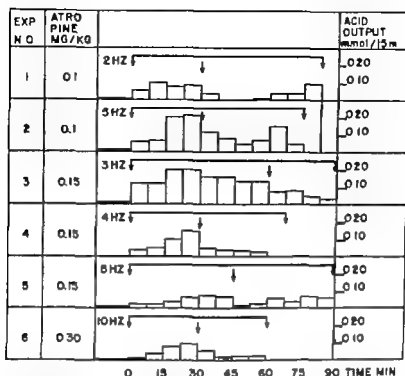


Fig. 1. 6 expts. demonstrating the inhibitory effect of atropine on vagally induced HCl secretion. Vagal stimulations were performed at 2-10 Hz. Atropine 0.1-0.3 mg/kg was administered when the HCl secretion had reached a plateau level and is indicated by an arrow in each expt (see also Table 1a).

TABLE 1a and 1b. Effect of atropine (0.1-0.3 mg/kg) (Table 1a) and Methamidate (4-20 mg/kg) (Table 1b) on vagally induced HCl secretion. A = Acid output 15 min before, B = 0-15 min and C = 15-30 min after the drugs were given. The secretion during periods B and C has been calculated in per cent of the control period prior to demonstration of atropine and methamidate (see also Fig. 1 and 2).

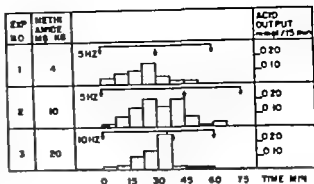
TABLE 1a

Exp. no.	Atropine mg/kg	A mmol	B		C	
			mmol	per cent of A	mmol	per cent of A
1	0.1	0.09	0.07	22	0.01	11
2	0.1	0.21	0.10	48	0.06	29
3	0.15	0.14	0.08	57	0.05	36
4	0.15	0.13	0.05	38	0.01	23
5	0.15	0.07	0.03	43	0.05	71
6	0.3	0.09	0.05	56	0.02	22

TABLE 1b

Exp. no.	Methamidate mg/kg	A mmol	B		C	
			mmol	per cent of A	mmol	per cent of A
1	4	0.13	0.05	38	0.04	15
2	10	0.18	0.05	28	0.03	17
3	20	0.2	0.03	15	0.01	5

3 experiments demonstrating the effect of methamide on induced HCl secretion. Stimulation was performed at 10 Hz. Methamide 10 mg/kg was administered. The HCl secretion had a plateau level (see also 1b).



II and II b Two successive vagal stimulations were performed at 2 or 5 Hz for 10 min in 5 cats (II a). In 10 other cats atropine in doses ranging from 0.5 mg/kg were given 10 min before the second stimulation (2 or 5 Hz) (Table II b). The gastric output following the second stimulation was expressed in per cent of the preceding control stimulation. The restimulation period was 20-30 min.

II

a Frequency Hz Gastric release induced by the second stimulation in per cent of the control stimulation (= 100 %)

5	115
5	95
5	103
5	119
5	115

110 per cent.

II b

a Frequency Hz Atropine mg/kg Gastric release induced by the second stimulation in per cent of control stimulation (= 100 %)

2	0	72	
2	0.5	97	
2	0.5	440	
2	0.6	183	
2	2.0	183	180
5	0.25	152	
5	0.25	83	
5	0.5	134	
5	1.0	158	
5	2.0	103	126

153 per cent.

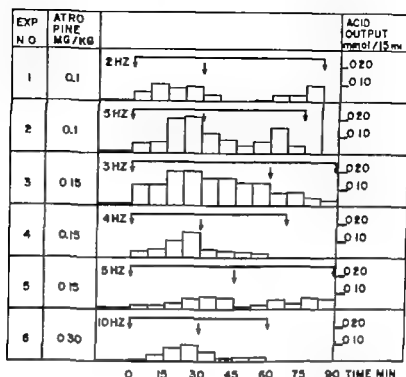


Fig. 1 6 expts. demonstrating the inhibitory effect of atropine on vagally induced HCl secretion. Vagal stimulation was performed at 2–10 Hz. Atropine 0.1–0.3 mg/kg was administered when the HCl secretion had reached a plateau level and is indicated by an arrow in each expt. (see also Table 1a).

TABLE 1a and 1b Effect of atropine (0.1–0.3 mg/kg) (Table 1a) and Methamidate (4–20 mg/kg) (Table 1b) on vagally induced HCl secretion. A = Acid output 15 min before, B = 0–15 min and C = 15–30 min after the drugs were given. The secretion during periods B and C has been calculated as per cent of the control period prior to administration of atropine and methamidate (see also Fig 1 and

TABLE 1

Exp. no.	Atropine mg/kg	A mmol	B		C	
			mmol	per cent of A	mmol	per cent of A
1	0.1	0.09	0.04	—	0.01	11
2	0.1	0.1	0.10	48	0.06	29
3	0.15	0.14	0.08	57	0.05	36
4	0.15	0.13	0.05	38	0.03	23
5	0.15	0.07	0.03	43	0.05	71
6	0.3	0.09	0.05	56	0.0	22

TABLE 1b

Exp. no.	Methamidate mg/kg	A mmol	B		C	
			mmol	per cent of A	mmol	per cent of A
1	4	0.13	0.05	38	0.02	15
2	10	0.18	0.05	28	0.01	17
3	20	0.2	0.03	15	0.01	5

Smith et al. 1972, Tepperman et al. 1973). It was surprising to find that in the present experiments atropine (0.2–2 mg/kg) was without effect on the vagally induced gastrin release. The results are in variance with those obtained in sham feeding experiment (*Nisvén et al. 1972* and *Tepperman et al. 1972*). In those expts. both gastrin release and HCl secretion were blocked by 0.2 mg/kg of atropine. However sham feeding induces vagal activation indirectly and most certainly several central neural pathways, which might be atropine sensitive or involved. Thus, since atropine passes the blood brain barrier the inhibiting effect of atropine on gastrin release is not necessarily exerted peripherally at the gastrin cell level, hence differences behind the atropine resistance are less probable, since *Smith et al. (1975)* tried to block vagally induced gastrin release in dog expts. similar to ours. Furthermore, the gastrin release induced by sham feeding in dogs is not abolished by a quaternary anticholinergic, which does not penetrate the blood brain barrier (*Ullrich-Wallentin and Andersen, to be published*).

Theoretically the administration of atropine might have caused higher antral pH during the period of vagally induced gastrin release through its inhibitory effect on HCl secretion. In atropine induced reduction of the gastrin release might in that case be masked by a simultaneous facilitation of the gastrin release by a higher antral pH. However the vagally induced gastrin peak appears within a few minutes after initiation of stimulation, when only insignificant amounts of HCl may have been produced, rendering such a hypothesis unlikely.

The inability of atropine to inhibit the vagally induced gastrin release might be explained in different ways. Either the vagi contain non-cholinergic neurons, which mediate the gastrin response or the fibres are cholinergic, but the receptors on the gastrin cells are not of the traditional atropine sensitive muscarinic type.

Other parasympathetic effects have been demonstrated to be atropine insensitive. *Martinsson (1972)* studied the effect of electrical vagal stimulation on gastric motility and tone. At certain stimulatory parameters a relaxation of the stomach was induced, which was only partly blocked by atropine, even when given in very high doses. Since the gastric relaxation was abolished when small amounts of ganglionic blockers were added, it was suggested that the partial inhibitory effect of atropine on the vagally induced gastric relaxation was exerted at the ganglionic level.

Adrenergic fibres are present in the vagi (*Lundberg et al. 1976*) and adrenergic nerves have been demonstrated within the antral mucosa (*Krokshin 1973*). Adrenergic fibres could thus be activated by electrical stimulation of the vagi. In humans, injections of adrenaline have been shown to induce a release of gastrin, which can be blocked by beta-adrenergic blocking substances (*Stadil and Rehfeld 1973*). However neither alpha nor beta-adrenergic blocking agents were able to inhibit the vagally induced gastrin release—rendering such a hypothesis unlikely (unpublished observations).

Since gastrin is released by antral perfusion with acetylcholine (*Jackson et al. 1972*) and also after i.v. infusion of this substance (*Booth et al. 1974*), the gastrin cells seem to be provided with receptors sensitive to acetylcholine. If these receptors correspond to the postganglionic parasympathetic nerve terminals, they are not of the ordinary atropine sensitive muscarinic type. However acetylcholine is not only postganglionic transmitter substance,

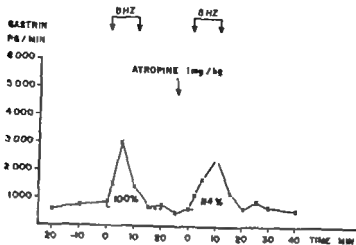


Fig. 3

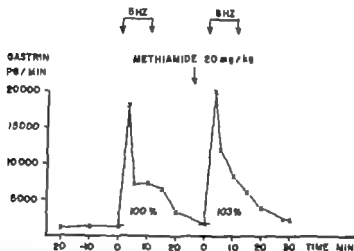


Fig. 4

Fig. 3 and 4 The experiment demonstrates the lack of effect of atropine (Fig. 3) and methiamide (Fig. 4) on vagally induced gastrin release. Two successive vagal stimulations were performed at 5 Hz for 10 min. Atropine (1 mg/kg) or methiamide 20 mg/kg was given 10 min before the second stimulation.

Discussion

The well established inhibitory effect of atropine on vagally induced gastric acid secretion was confirmed by the present expts. Methiamide, a histamine-2 receptor antagonist was an even more potent inhibitor in the dosage range tested. Neither atropine, nor methiamide blocked the gastrin release caused by vagal stimulation. In other words, neither of the drugs inhibit the gastric secretory response by inhibition of gastrin release.

Atropine (0.1–0.3 mg/kg) reduced the acid secretory response to vagal stimulation by more than 50%. Similar doses have been shown to almost abolish the HCl secretion induced by sham feeding in dogs (Nilsson *et al* 1972, Tepperman *et al* 1972).

Since vagally induced gastrin release is supposed to be mediated via cholinergic fibres

son *et al.* 1972, Tepperman *et al.* 1972). It was surprising to find that in the present experiments atropine (0.2–2 mg/kg) was without effect on the vagally induced gastrin release. The results are at variance with those obtained in sham feeding experiments (Nilsson *et al.* 1972 and Tepperman *et al.* 1972). In those expts. both gastrin release and HCl secretion were elicited by 0.2 mg/kg of atropine. However sham feeding induces vagal activation indirectly and most certainly several central neural pathways, which might be atropine sensitive. Thus, since atropine passes the blood-brain barrier the inhibiting effect of atropine on gastrin release is not necessarily exerted peripherally at the gastrin cell level. Possible differences behind the atropine resistance are less probable, since Smith *et al.* (1975) failed to block vagally induced gastrin release in dog expts. similar to ours. Furthermore, the gastrin release induced by sham feeding in dogs is not abolished by a quaternary anti-cholinergic, which does not penetrate the blood-brain barrier (Uvnäs-Wallensten and Andersson, to be published).

Theoretically the administration of atropine might have caused a higher antral pH during a period of vagally induced gastrin release through its inhibitory effect on HCl secretion. A atropine induced reduction of the gastrin release might in that case be masked by a reflexogenic facilitation of the gastrin release by a higher antral pH. However the vagally induced gastrin peak appears within a few minutes after initiation of stimulation, when only significant amounts of HCl may have been produced, rendering such a hypothesis unlikely.

The inability of atropine to inhibit the vagally induced gastrin release might be explained in different ways. Either the vagi contain non-cholinergic neurons, which mediate the gastrin response or the fibres are cholinergic, but the receptors on the gastrin cells are not of the additional atropine sensitive muscarinic type.

Other parasympathetic effects have been demonstrated to be atropine insensitive. Martenson (1972) studied the effect of electrical vagal stimulation on gastric motility and tone. At certain stimulatory parameters a relaxation of the stomach was induced, which was only partly blocked by atropine, even when given in very high doses. Since the gastric relaxation was abolished when small amounts of ganglionic blockers were added, it was suggested that the partial inhibitory effect of atropine on the vagally induced gastric relaxation was exerted at the ganglionic level.

Adrenergic fibres are present in the vagi (Lundberg *et al.* 1976) and adrenergic nerves have been demonstrated within the antral mucosa (Krukshina 1973). Adrenergic fibres could then be activated by electrical stimulation of the vagi. In humans, injections of adrenaline have been shown to induce a release of gastrin, which can be blocked by beta-adrenergic blocking substances (Stadil and Rehfeld 1973). However neither alpha- nor beta-adrenergic blocking agents were able to inhibit the vagally induced gastrin release—rendering such a hypothesis unlikely (unpublished observations).

Since gastrin is released by antral perfusion with acetylcholine (Jackson *et al.* 1972) and also after i.v. infusion of this substance (Booth *et al.* 1974), the gastrin cells seem to be provided with receptors sensitive to acetylcholine. If these receptors correspond to the postganglionic parasympathetic nerve terminals, they are not of the ordinary atropine sensitive muscarinic type. However acetylcholine is not only a postganglionic transmitter substance,

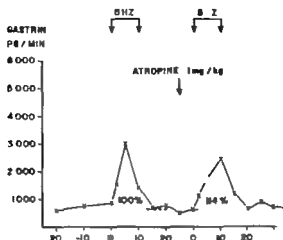


Fig. 3

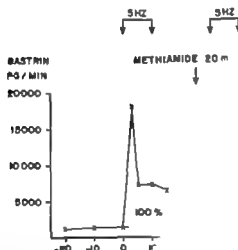


Fig 3 and 4 The experiment demonstrates on vagally induced gastrin release. Two ml Atropine (1 mg/kg) or methamidate 20 mg

The well established inhibitor was confirmed by the presence of an even more potent inhibitor which blocked the gastrin release and inhibit the gastric secretion.

Atropine (0.1-0.3 mg) more than 50%. Similar by sham feeding in d. Since vagally induced

Effect of Hypercapnia and Hypocapnia on Tryptophan and Tyrosine Hydroxylation in Rat Brain

By

A. CARLSSON, T. HOLMÉN, M. LINDBQVIST and B. K. SUNDÖ

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Abstract

CARLSSON, A., T. HOLMÉN, M. LINDBQVIST and B. K. SUNDÖ. *Effect of hypercapnia and hypocapnia on tryptophan and tyrosine hydroxylation in rat brain*. Acta physiol. scand. 1977 99 503-509.

The effects of induced hypo- and hypercapnia upon the rate of hydroxylation of tryptophan and tyrosine in the rat brain are studied by measuring the accumulation of 5-HTP and DOPA following administration of the aromatic L-aminoacid decarboxylase inhibitor 3-hydroxybenzylhydrazine HCl (NSD 1015). The results suggest that the hydroxylation of tryptophan varies directly with the tissue P_{O_2} . On the other hand, the hydroxylation of tyrosine did not show a simple relationship to P_{O_2} but appeared to be influenced by pH changes.

The rate-limiting enzymes in the synthesis of catechol and indole amines are tyrosine and tryptophan hydroxylase, respectively. These enzymes are oxygen dependent and their activities vary with the P_{O_2} *in vivo* (Fisher and Kaufman 1972, Green and Sawyer 1966). Recently it was shown that administration of gas mixtures with low oxygen content reduces the rate of hydroxylation of tyrosine and tryptophan *in vivo* (Davis and Carlsson 1973 a and b) and, for tryptophan, the rate of hydroxylation was found to change linearly with the arterial venous P_{O_2} (Davis *et al.* 1973). The results suggest that the rate of synthesis of amines is related to tissue P_{O_2} . However, hypoxia *in vivo* induces other changes in the tissue than a mere reduction of P_{O_2} . For example, it should be recalled that although the cerebral energy state is upheld at the oxygen tensions previously used to study monoamine metabolism, cellular acidosis develops at arterial P_{O_2} values below about 50 mmHg (Siesjö and Nilsson 1971, MacMillan and Siesjö 1972, Norberg and Siesjö 1975). Previous studies indicate that hypercapnia may reverse the effect of hypoxia on catecholamine but not on 5-hydroxytryptamine (5-HT) synthesis in the rat brain (Brown *et al.* 1974).

In the present experiments we have studied the effects of induced hypocapnia and hypercapnia upon the rate of hydroxylation of tryptophan and tyrosine in the rat brain, employing a technique in which the accumulation of 5-HTP and DOPA was measured following administration of the aromatic L-aminoacid decarboxylase inhibitor 3-hydroxybenzylhydrazine

it is also an intraganglionic transmitter. Assuming that the gastrin cells serve as the postganglionic neurons of endocrine parasympathetic nerves (in analogy with the noradrenaline and adrenaline containing cells in the adrenal medulla) the cholinergic receptors could be of the nicotinic type.

In conclusion the vagal neuro-effector junction at the antral G-cells differ from those on the HCl glands. Either the vagal fibres are not cholinergic—or they are cholinergic—but then the G-cell receptors are not of the muscarinic type.

The investigation has been supported by grants from Magnus Bergvalls Stiftelse

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Table I. Body temperature, mean arterial blood pressure, hemoglobin concentration and acid-base balance of arterial blood in the different groups of rats.

group	Temp (°C)	MABP (mmHg)	Hb (g/100 ml)	P _{a,CO₂} (mmHg)	pH	B.E. (mmol/l)
normocapnia SD 1015	37.2 ±0.1	133 ±7	15.1 ±0.6	41.1 ±0.7	7.371 ±0.019	0.6 ±1.1
normocapnia SD 1015	37.0 ±0.2	137 ±8	15.3 ±0.2	44.7 ±0.4	7.438 ±0.017	0.8 ±0.7
hypercapnia SD 1015	37.1 ±0.0	129 ±3	15.1 ±0.4	82.9 ±0.9	7.178 ±0.009	4.7 ±0.6
hypercapnia hypoxia SD 1015	37.3 0.1	133 ±5	15.1 ±0.5	84.6 ±1.0	7.115 ±0.018	4.6 ±1.4

Values are means ± S.E. for groups of 4-6 rats.

by Adkinson and Walton (1967). Statistical differences were evaluated using Wilcoxon rank-sum test, t-test for the normocapnic data in Table IV where one-way analysis of variance followed by t-test was used (McNemar, 1962).

Results

Physiological variables

Table I shows body temperature, mean arterial blood pressure (MABP), hemoglobin concentration (Hb), arterial P_{CO₂}, and pH, and the calculated base excess concentration (B.E.). Body temperature was close to 37°C in all groups. Every animal had a mean arterial blood pressure of 120 mmHg, or higher and a hemoglobin concentration exceeding 13.2 g (100 ml)⁻¹. In the hypocapnic group arterial P_{CO₂} was close to 15 mmHg, and in the hypercapnic groups close to 85 mmHg. Apart from the fact that hypercapnia gave rise to a moderate respiratory acidosis arterial plasma pH changed according to the variation in P_{CO₂}. Table II illustrates the arterial and cerebral venous P_{O₂} values. The arterial P_{O₂} was close to 100 mmHg except in the second hypercapnic group in which it was deliberately reduced. In hypocapnia and (normoxic) hypercapnia there was a highly significant decrease and in-

Table II. Oxygen tensions of arterial as well as cerebral venous blood of rats in the different groups.

group	P _{a,O₂}	P _{c,O₂}
normocapnia SD 1015	104 ±4	39.1 ±1.0
normocapnia SD 1015	101 ±6	18.5 ±2.1
hypercapnia SD 1015	90 ±2	60.0 ±1.7
hypercapnia hypoxia SD 1015	47.6 ±2.5	38.3 ±1.5

P_c (mmHg) for groups of 4-6 animals.

HCl (NSD 1015). The experiments had the dual objective of studying the influence of changes in tissue P_{O_2} and in intracellular pH. With the degree of hypocapnia induced (arterial P_{CO_2} about 15 mmHg) there is a reduction in venous (and tissue) P_{O_2} but, due to a stimulation of anaerobic glycolysis, changes in intracellular pH (pH_i) are either small or absent (see Mac Millan and Siesjö 1973). Hypercapnia induced under normoxic condition causes 2 main changes: (1) a fall in pH_i and (2) an increase in venous (and tissue) P_{O_2} due to a concomitant rise in cerebral blood flow. In order to separate the effects of hyperoxia and acidosis a second hypercapnic group was studied in which venous P_{O_2} was reduced to normal by the administration of a gas mixture with low oxygen content.

Methods

Operative and sampling technique

Male Wistar rats, weighing 270–370 g, were used for the experiments. Anaesthesia was induced with halothane. The rats were then immobilized with t. bucarine chloride, administered *i.p.* quickly tracheotomized and ventilated with a Starling type respirator with an inflow of 70% N_2O and 30% O_2 . The right femoral artery was cannulated for electromanometric blood pressure measurements and 100- μ l samples of blood were drawn for analysis of P_{O_2} , P_{CO_2} , pH and hemoglobin content. A skin incision was made over the skull bone, and a burr hole was placed over the superior sagittal sinus for sampling of cerebral venous blood. Two samples of blood from the femoral artery were drawn for measurements of pH, P_{CO_2} and P_{O_2} . It was controlled that a P_{CO_2} constancy of better than 10% between the two samples was obtained. The rectal temperature was kept close to 37°C during the experiment. 4 groups of animals were used.

Group I Normocapnia. The respirator was adjusted to give arterial CO_2 tensions of about 35–40 mmHg and arterial O_2 tensions of about 100 mmHg. 30 min before the end of the experiment NSD 1015 was administered *i.p.* (100 mg kg^{-1}). 15 and 29 min after the administration of NSD 1015, P_{O_2} , P_{CO_2} and pH were measured in arterial and cerebral venous blood. Thirty min after the administration of NSD 1015 liquid nitrogen was poured into a plastic funnel fitted into the skin incision over the skull bone thereby freezing the brain *in situ*. The frozen brain was split into two parts. Cortical tissue was taken from the right half of the brain for subsequent enzymatic, fluorometric analyses of glucose, glucose-6-phosphate, lactate, pyruvate, citrate, α -ketoglutarate, malate, ammonia, glutamate, glutamine, aspartate, phosphocreatine, ATP, ADP and AMP. The left hemisphere was analysed for tryptophan (TRY), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), tyrosine (TYR), dihydroxyphenylalanine (DOPA), dopamine (DA) and noradrenaline (NA).

Group II Hypocapnia. 5 min before the administration of NSD 1015, hyperventilation was induced to give arterial CO_2 tensions of about 15 mmHg. The animals were otherwise treated as were the animals in group I.

Group III Hypercapnia. 5 min before the administration of NSD 1015 CO_2 was added to the inspired gas. Hyperventilation was induced, N_2O was withdrawn and the final composition of the inspired gas mixture was about 8% CO_2 and 25% O_2 in N_2 . The arterial CO_2 tensions in this group varied between 80 and 85 mmHg. With the exception of the induced hypercapnia the animals were treated as group II.

Group IV Hypercapnia associated with hypoxia. In this group the inspired gas mixture consisted of CO_2 , O_2 and N_2 . In order to get arterial O_2 tensions of about 50 mmHg and no reduction in cerebral venous O_2 tensions, compared with normal animals, the O_2 content of the inspired gas mixture was reduced to about 15%. Otherwise the group was identical to group III.

Analytical techniques. P_{O_2} , P_{CO_2} and pH were measured at 37°C with microelectrodes (Eckwerter and Co. Kiel Radiometer Copenhagen). Hemoglobin concentration was measured photometrically (Vitatron). For measurements of organic phosphates, carbohydrate metabolites, ammonia, and amino acids, the tissue was extracted at $-24^\circ C$ and enzymatic, fluorometric techniques were used (for details, see Folbergrovi *et al.* 1972 and b, 1974). The methods for tyrosine, tryptophan, monoamines and 5-HIAA have been described previously (see Davis and Carlsson 1973 b).

Calculations. The blood base excess values were derived from the P_{CO_2} , the pH and the hemoglobin concentration according to the equation given by Siggaard Andersen (1966). The energy state of the brain was evaluated from the energy charge of the adenosine nucleotide system (EC), calculated by the formula

TABLE II. Contents of tryptophan (TRY), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-acetic acid (5-HIAA), tyrosine (TYR), dihydroxyphenylalanine (DOPA), dopamine (DA), and noradrenaline (NA) in the left part of the brain of rats in different groups.

Exp group	TRY	5-HTP	5-HT	5-HIAA	TYR	DOPA	DA	NA
Normocapnia	68	142	796	217	16.7	236	1179	47
ASD 10/15	± 0.3	± 16	± 31	± 11	± 0.7	± 4	± 113	± 16
Hypocapnia	7.2	111	333	195	14.1	196	1177	780
ASD 10/15	± 0.4	± 4	± 23	± 7	± 0.8	± 13	± 125	± 7
Hypercapnia	7.4	177	360	247	14.9	796	1185	274
ASD 10/15	± 0.4	± 13	± 40	± 21	± 0.7	± 14	± 68	± 27
Normocapnia	7.8	148	292	16	14.5	341	1196	193
ASD 10/15	± 0.6	± 7	± 4	± 15	± 1.4	± 1	± 171	± 27

Values are means \pm S.E. (μ g/g wet wt for TRY and TYR, otherwise ng/g wet wt) for groups of 6-7 rats. Values from the normocapnia group: * $p < 0.05$ ** $p < 0.001$.

from P_{O_2} and tyrosine hydroxylation. However when arterial P_{O_2} was normalized under eucapnic conditions DOPA accumulation did not decrease. Thus, hydroxylation of tyrosine does not seem to be a simple function of venous P_{O_2} but must be influenced by pH (see Discussion).

Discussion

On discussing the results on monoamine metabolism it seems warranted to recall some of the changes induced in the tissues by hypocapnia and hypercapnia. Since cerebral blood flow varies directly with the CO₂ tension, and since variations in the CO₂ tension between 15 and 85 mmHg do not lead to changes in the cerebral oxygen uptake (for data on rats, see

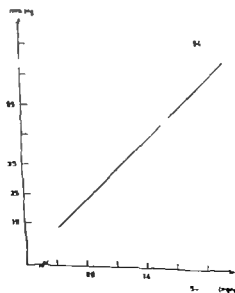


Fig. 1. Relation between oxygen tension in arterial blood and contents of 5-HTP in the brain of rats. The filled circles denote mean values in the different groups of animals. Correlation coefficient.

TABLE III. Contents of phosphocreatine (PCr), creatine (Cr), ATP, ADP and AMP as well as adenylate energy charge (EC) in the different groups of rats.

Exp. group	PCr	Cr	ATP	ADP	AMP	EC
Normocapnia	4.58	5.58	3.05	0.277	0.023	0.957
NSD 1015	± 0.10	± 0.03	± 0.02	± 0.005	± 0.001	± 0.001
Hypocapnia	4.78	5.74	3.14	0.302	0.024	0.950
NSD 1015	± 0.13	± 0.03	± 0.01	± 0.003	± 0.001	± 0.001
Hypercapnia	4.38	5.98	3.15	0.274	0.022	0.934
NSD 1015	± 0.03	± 0.04	± 0.04	± 0.002	± 0.001	± 0.000
Hypercapnia + hypoxia	4.28	6.14	3.13	0.280	0.021	0.953
NSD 1015	± 0.11	± 0.09	± 0.02	± 0.002	± 0.001	± 0.001

Values are means \pm S.E. ($\mu\text{mol/g wet wt}$) for groups of 4-6 rats.

Differs from the normocapnia group $\eta < 0.05$ $\rho < 0.02$.

crease in venous P_{O_2} , respectively. In the hypoxic hypercapnic group the venous P_{O_2} was close to normal.

Organic phosphates, carbohydrate metabolites, ammonia and amino acids

These measurements were carried out to secure that the administration of NSD 1015 did not by itself influence the metabolic state of the tissue. Table III gives the values for PCr, creatine, ATP, ADP, AMP and adenylate energy charge. The normocapnic group given NSD 1015 had values for these labile substances that were very close to those previously obtained in the laboratory (*cf.* Folbergrova *et al.* 1972a, MacMillan and Siesjö 1973), and we conclude that NSD 1015 does not influence cerebral energy state (*cf.* Davis *et al.* 1973). There was a small rise in ATP content in the hypocapnic and hypercapnic groups but the adenylate energy charge did not differ between the groups. In the hypocapnic group, there was a small increase in ADP but this increase can be observed also in animals who have not received NSD 1015 (MacMillan and Siesjö 1973). We conclude that all groups studied had an essentially normal energy state and that the presence of NSD 1015 did not influence energy balance during the hypocapnic and hypercapnic conditions.

Analysis of tissue concentrations of glucose, glucose-6-phosphate, pyruvate, lactate, citrate, α -ketoglutarate, malate, glutamate, glutamine, aspartate and ammonia showed that NSD 1015 had no significant effect on carbohydrate metabolites, amino acids or ammonia under normocapnic, hypocapnic or hypercapnic conditions.

Monoamine metabolism

The values are given in Table IV. Since all other variables remained essentially constant in the various groups, attention should be directed towards changes in the concentrations of 5-HTP and DOPA. 5-HTP and DOPA concentrations were decreased in hypocapnia and increased in hypercapnia. Statistical significance was, however, not reached for the slight DOPA induced decrease in hypocapnia. 5-HTP formation was normalized when P_{O_2} was lowered during hypercapnia. Thus, hydroxylation of tryptophan seems to vary directly with the venous P_{O_2} (Fig. 1). Under normoxic conditions a similar relationship

TABLE IV. Contents of tryptophan (TRY), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-acetic acid (5-HIAA), tyrosine (TYR), dihydroxyphenylalanine (DOPA), dopamine (DA), and noradrenaline (NA) in the left part of the brain of rats in different groups.

rat group	TRY	5-HTP	5-HT	5-HIAA	TYR	DOPA	DA	NA
normocapnia SD 1015	118 ± 0.3	142 ± 16	298 ± 31	17 ± 11	16.7 ± 0.7	36 ± 4	1 179 ± 113	47 ± 16
hypocapnia SD 1015	72 ± 0.4	83 ± 4	333 ± 22	193 ± 7	14.1 ± 0.8	196 ± 13	1 277 ± 125	70 ± 7
hypercapnia SD 1015	74 ± 0.4	177 ± 13	360 ± 40	247 ± 21	14.9 ± 0.7	296 ± 14	1 185 ± 68	274 ± 27
hypocapnia + hypoxia SD 1015	78 ± 0.6	148 ± 7	292 ± 24	216 ± 13	14.5 ± 1.4	341 ± 12	1 196 ± 171	193 ± 7

Values are means ± S.E. (arbitrary wet wt for TRY and TYR, otherwise ng/g wet wt) for groups of 6-7 rats. Different from the normocapnia group, *p < 0.05, **p < 0.001.

between P_{O_2} and tyrosine hydroxylation. However when venous P_{O_2} was normalized under hypercapnic conditions DOPA accumulation did not decrease. Thus, hydroxylation of tyrosine does not seem to be a simple function of venous P_{O_2} but must be influenced by pH as well (see Discussion).

Discussion

Before discussing the results on monoamine metabolism it seems warranted to recall some of the changes induced in the tissue by hypocapnia and hypercapnia. Since cerebral blood flow varies directly with the CO₂ tension, and since variations in the CO₂ tension between 15 and 85 mmHg do not lead to changes in the cerebral oxygen uptake (for data on rats, see

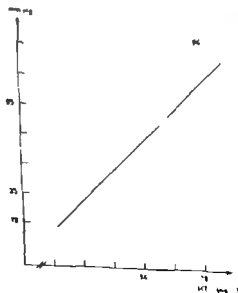


Fig. 1. Relation between oxygen tension in cerebral venous blood and contents of 5-HTP in brain tissue of rats. The filled circles denote mean values in the different groups of animals. $r = 0.96$.

Eklöf *et al* 1973) It follows that hypocapnia is accompanied by decreased and hypercapnia by increased P_{O_2} in brain tissue. In view of the results reported by Davis and Carlsson (1973a and b see also Davis *et al* 1973) it would be expected that hypocapnia should decrease and hypercapnia increase the rate of hydroxylation of tyrosine and tryptophan. However, this is so only if the accompanying acid-base changes do not influence the hydroxylation. Hypercapnia is invariably associated with a decrease in intra- and extracellular pH of the brain (see Siesjö *et al* 1972). In order to differentiate between effects due to hyperoxia and acidosis arterial P_{O_2} was reduced in one hypercapnic group so as to normalize cerebral venous P_{O_2} . A decrease in P_{CO_2} , i.e. hypocapnia, increases intra- and extracellular pH. However, when P_{aCO_2} is reduced to about 15 mmHg there seems to be sufficient stimulation of anaerobic glycolysis to achieve normalization of pH_i (MacMillan and Siesjö 1973). Thus, in the present hypocapnic group there should have been a decrease in tissue P_{O_2} but not only a very small change in pH_i .

In the present experiments, the tissue concentrations of tryptophan, 5-HTP, 5-HT, 5-HIAA, tyrosine, DOPA, dopamine and noradrenaline were measured in animals given NSD 1015. The results suggest that the hydroxylation of tryptophan, i.e. the first step in the synthesis of 5-HT, varies directly with the tissue P_{O_2} and that pH changes have little influence. In contrast, the hydroxylation of tyrosine did not show a simple relationship to P_{O_2} . The results suggest that pH changes contribute to the increased rate of synthesis of DOPA under hypercapnic conditions. That a difference in response to pH changes exists between the hydroxylation of tryptophan and of tyrosine is also supported by the data of Brown *et al* (1974 see Introduction).

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Sutter, M. C. and B. Ujeng

Contractility, Muscle Mass and Agonist Sensitivity of Isolated Portal Veins from Normal and Hypertensive Rats

Ureda-Wasboretan, K. and H. Andersson

Effect of Acropine and Met-formide on Vagally Induced Gastric Acid Secretion and Gastrin Release in Anesthetized Cats

Carlsson, A., T. Hökfelt, M. Lindqvist and B. K. Sverijö

Effect of Hypercapnia and Hypocapnia on Tryptophan and Tyrosine Hydroxylase in Rat Brain

Art. Commercialization

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Increase in Gastric Concentration in the Duodenal Mucosa of Dogs Following Reabsorption of the Basic Anesthetics

Supplement appended

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Units	Symbols
kilogramme	kg
second, millisecond	s, ms
mole, millimole, micromole, nanomole	mol, mmol, μ mol
picomole	nmol, pmol
meter, millimeter	m, mm
micrometer	μ m
nanometer	nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N ($kg \cdot m/s^2$)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J ($N \cdot m$)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd \cdot sr)
lux (illumination)	lx (lm/m^2)

Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J) ^a
(kilopond)	kp (9.81 N)
(millimeters of mercur)	mm Hg
(curry)	(1.333 bar) ^a
(millibar)	mbar (100 Pa) ^a
curie	Cl
liter, milliliter, microliter	l, ml, μ l
degree Celsius	°C

Conversion factors to be given in Methods.

